Division of Nephrology
Department of Medicine
Helsinki University Central Hospital

Folkhälsan Institute of Genetics
Folkhälsan Research Center

Research Programs Unit
Diabetes and Obesity
University of Helsinki
Helsinki, Finland

Doctoral Program in Biomedicine
Doctoral School in Health Sciences
Department of Medicine
University of Helsinki
Helsinki, Finland

Biological factors involved in the modulation of bacterial endotoxin-mediated inflammation in type 1 diabetes

Christopher L. Fogarty

Academic Dissertation

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Supervisors

Docent Markku Lehto
Division of Nephrology
University of Helsinki and Helsinki University Central Hospital
Helsinki, Finland

Folkhälsan Institute of Genetics
Folkhälsan Research Center, Helsinki, Finland

and

Professor Per-Henrik Groop
Division of Nephrology
University of Helsinki and Helsinki University Central Hospital
Helsinki, Finland

Folkhälsan Institute of Genetics
Folkhälsan Research Center, Helsinki, Finland

Reviewers

Docent Katariina Öorni
Wihuri Research Institute, Helsinki, Finland

Krister Wennerberg
Institute for Molecular Medicine Finland
University of Helsinki
Helsinki, Finland

Opponent

Peter Hamar
Semmelweis University
Budapest, Hungary

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Contents

Definitions:

To maintain the readability of this dissertation, the use of abbreviations was minimized. Those that are used are found below:

AER – Albumin excretion rate

AGE – Advanced glycosylated end products

AP – Alkaline phosphatase

APC – Antigen presenting cells

BMI – Body mass index

CRP – C-reactive protein

eGFR – Estimated glomerular filtration rate

ESRD – End stage renal disease

HDL – High density lipoprotein

IBD – Inflammatory bowel disease

ICAM – Intercellular adhesion molecule

IFN (α&γ) – Interferon α&γ

IL – Interleukin

LAL – Limulus amebocyte lysate

LADA – Latent autoimmune diabetes in adults

LPS – Lipopolysaccharide

MBL – Mannose-binding lectin

mDC – Myeloid dendritic cell

MetS – Metabolic syndrome

MODY – Maturity onset diabetes of the young

NF-kB – Nuclear factor kB

PAMP – Pathogen- associated molecular pattern

PRR – Pattern recognition receptor

RTPCR – Reverse transcription polymerase chain reaction

SCFA – Short chain fatty acid

T1D – Type 1 Diabetes

T2D – Type 2 diabetes

TLR – Toll-like receptor

TNF – Tumor necrosis factor

WHO – World Health Organization

HbA1c – Glycated hemoglobin

List of Original Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III):

I. Peräneva, L., **Fogarty, C. L.**, Pussinen, P. J., Forsblom, C., Groop, P. H., & Lehto, M. Systemic exposure to Pseudomonal bacteria: a potential link between type 1 diabetes and chronic inflammation. Acta Diabetologica (2013) 50:351-361.

II. **Fogarty, C. L.**, Nieminen, J. K., Peräneva, L., Lassenius, M. I., Taskinen, M.-R., Jauhiainen, M., Kirveskari, J., Pussinen, P., Hörkkö, S., Mäkinen, V.-P., Gordin, D., Forsblom, C., Groop, P.-H., Vaarala, O., and Lehto, M. High fat meal induces systemic cytokine release without evidence of endotoxemia-mediated cytokine production from circulating monocytes and myeloid dendritic cells. Acta Diabetologica (2015) 52(2):315-22.

III. Lassenius, M.I.*, **Fogarty, C. L.***, Blaut, M., Haimila, K., Riittinen, L., Paju, A., Kirveskari, J., Järvelä, J., Ahola, A. J., Gordin, D., Kumar, A., Hamarneh, S. R., Hodin, R., Sorsa, T., Tervahartiala, T., Hörkkö, S., Pussinen, P., Forsblom, C., Jauhiainen, M., Taskinen, M-R., Groop, P.-H., Lehto, M. on behalf of the FinnDiane Study. Intestinal alkaline phosphatase at the crossroad of intestinal health and disease: a putative role in type 1 diabetes. J Intern Med (2017) 281(6):586-600.

*Equal Contribution

Contribution

I. Participated in data analysis and interpretation and the writing of the manuscript. Performed a significant portion of the DNA isolation, PCR amplification, cloning and sequencing work, downstream data processing and BLAST searches, and the RT-PCR quantification of bacterial DNA.

II. Participated in the study design, data analysis and interpretation and manuscript writing. Performed the flow cytometry experiments and analysis as well as the cytokine multiplex experiments.

III. Participated in study design, data analysis and interpretation and manuscript writing.
Study III was also included in the thesis of Mariann Lassenius.

Publications not included in this thesis

1. Duennwald, T., Bernardi, L., Gordin, D., Sandelin, A., Syreeni, A., **Fogarty, C.**, Kytö JP, Gatterer H, Lehto M, Hörkkö S, Forsblom C, Burtscher M, Groop, P. H. Effects of a Single Bout of Interval Hypoxia on Cardio-Respiratory Control in Patients with Type 1 Diabetes Mellitus. Diabetes (2013) 62:4220-4227.

2. Lassenius, M.I., Mäkinen, V.-P., **Fogarty, C. L.**, Peräneva, L., Jauhiainen, M., Pussinen, P.J., Taskinen, M.-R., Kirveskari, J., Vaarala, O., Nieminen, J.K., Hörkkö, S., Kangas, A.J., Soininen, P., Ala-Korpela, M., Gordin, D., Ahola, A.J., Forsblom, C., Groop, P.-H., Lehto, M. Patients with type 1 diabetes show signs of vascular dysfunction in response to multiple high-fat meals. Nutr Metab (Lond). (2014) 11:28.

3. Saurus, P., Kuusela, S., Lehtonen, E., Hyvönen, M. E., Ristola, M., **Fogarty, C. L.**, Tienari, J., Lassenius, M. I., Forsblom, C., Lehto, M., Saleem, M.A., Groop, P-H., Holthöfer, H., Lehtonen S. Podocyte apoptosis is prevented by blocking the Toll-like receptor pathway. Cell Death and Disease (2015) May 7;6:e1752.

4. Haapaniemi, E. M., **Fogarty, C. L.**, Keskitalo, S., Takayama, S., Ilander, M., Krjutshkov, K., Vihinen, H., Jokitalo, E., Mustjoki, S., Lehto, M., Hautala, T., Varjosalo, M., Velagapudi, V., Seppänen, M., Kere, J. Combined immunodeficiency with hypoglycemia caused by mutations in hypoxia up-regulated 1. The Journal of Allergy and Clinical Immunology (2016).

5. Chen, W., Roslund, K., **Fogarty, C.L.,** Pussinen, P., Halonen, L., Lehto, M., Groop, P.H., Metsälä, M., Lehto, M. Detection of hydrogen cyanide from the oral pathogen Porphyromonas gingivalis by cavity ring down spectroscopy. Scientific Reports (2016) March 4;6:22577.

6. Saurus, P., Dumont, V., Kuusela, S., Lehtonen, E., **Fogarty, C. L.**, Lassenius, M. I., Forsblom, C., Lehto, M., Saleem, M. A., Groop, P.-H., Holthöfer, H., and Lehtonen, S. CDK2 protects podocytes from apoptosis and is downregulated in podocyte injury. Scientific Reports (2016) Feb 15;6:21664.

7. Wasik, A. A., Dumont, V., Tienari, J., Nyman, T. A., **Fogarty, C. L.,** Forsblom, C., Lehto, M., Groop, P-H., and Lehtonen, S. Septin 7 and nonmuscle myosin IIA compete for binding to the SNARE complex to regulate glucose uptake into podocytes. Experimental Cell Research (2017) 350(2):336-348.

8. Kaustio, M., Haapaniemi, E., Nurkkala, H., Park G., Syrjänen, J., Einarsdottir, E., Sahu, B., Kilpinen, S., Rounioja, S., **Fogarty, C.L.,** Glumoff, V., Kulmala, P., Katayama, S., Tamene, F., Trotta, L., Morgunova, E., Krjutskov, K., Anssi, L., Martelius, T., Helminen, M., Mustjoki, S., Taipale, J., Saarela, J., Kere, J., Varjosalo, M., and Seppänen, M. Damaging heterozygous mutations in NFKB1 lead to diverse immunologic phenotypes. The Journal of Allergy and Clinical Immunology (2017).

Abstract

Background

Type 1 diabetes (T1D) is a disease characterized by the autoimmune destruction of insulin-producing pancreatic β cells. Diabetic nephropathy is a life-threatening complication of T1D, characterized by the progressive loss of kidney function. Approximately one-third of all patients with T1D develop diabetic nephropathy. Bacterial DNA and bacterial lipopolysaccharides (LPS) are two categories of bacterial remnants that are known to induce inflammation. Inflammation and elevated levels of bacterial remnants have previously been shown to be associated with the development of diabetic nephropathy, and there is evidence in mice that these factors play a causal role in disease progression.

The general aim of this thesis is to identify biological factors that modulate bacterial remnant-mediated inflammation in patients with T1D. Specifically, we aimed to better understand the composition, origin and consequences of bacterial remnants in circulation in the context of T1D by (1) evaluating the presence of bacterial DNA in the sera of patients with T1D and controls (Study I) and (2) measuring LPS activity, inflammation, inflammatory potential and gut-related factors in the context of multiple high-fat meals given to patients with T1D and healthy controls (Studies II & III).

Study I found a higher frequency of Pseudomonal (Pa) DNA in circulation as well as elevated anti-Pa IgA levels in patients with T1D. These Pa-specific IgA antibodies correlated with higher C-reactive protein, a marker for inflammation, suggesting that patients with T1D undergo recurrent or chronic Pseudomonal exposure and potentially explaining the chronic inflammation in patients with T1D.

Contrary to our hypotheses, study I found no correlation between LPS activity and either bacterial DNA composition or antibodies against identified bacterial species. This may be attributable to differences in the half-lives and host clearance mechanisms of bacterial remnants. However, it is also possible that the entry mechanisms and points of entry for LPS and bacterial DNA are different.

The identification of numerous bacteria known to be present in the oral cavity suggests that one possible point of entry is the oral cavity.

Another possible point of entry for bacterial remnants is the gut. Circulating LPS was previously shown to increase after the ingestion of high-fat meals by healthy adults. Study II found a pronounced increase in serum markers of inflammation after multiple high-fat meals; however, our data suggest this inflammation was not attributable to increases in circulating LPS. Indeed, circulating LPS levels appeared to have no effect on immune cell activation or systemic inflammation. This led us to investigate factors related to intestinal homeostasis, particularly focusing on factors such as alkaline phosphatases, which might affect the potency of intestinally derived LPS. In Study III, we found a general disturbance in factors related to gut homeostasis in patients with T1D. Specifically, low levels of fecal alkaline phosphatase found in patients with T1D contributed to increased LPS potency in the intestine, which in turn boosted intestinal inflammation.

These studies help shed light on the potential routes of entry for bacterial remnants and the possible mechanisms underlying the inflammatory response induced by high-fat meals.

1 Introduction

Finland has the highest incidence of type 1 diabetes (T1D) worldwide, with approximately 30,000 currently diagnosed individuals within a population of just over 5 million[1].

It is estimated that one-third of T1D patients develop renal disease within 20 years after disease onset[2]. Microvascular and macrovascular complications such as neuropathy, nephropathy, retinopathy, atherosclerosis and stroke are frequently observed in T1D patients with long disease duration and metabolic disturbance. Diabetic complications such as cardiovascular disease, atherosclerosis and nephropathy have been associated with elevated levels of inflammatory markers[3-5]. Continuous exposure to microbial agents partially explains the chronic inflammation reported in patients with T1D. Two microbial agents known to induce inflammation are bacterial DNA and endotoxin.

The bacterial endotoxin lipopolysaccharide (LPS) is a unique glycolipid that serves as a component of the outer membrane in Gram-negative bacteria. In two recent studies, we reported that LPS activity is positively associated with inflammatory markers and the advancement of diabetic nephropathy[6,7]. Therefore, elevated levels of LPS may play a key role in the promotion and progression of diabetic complications. A better understanding of the origin and consequences of circulating LPS will help inform the development of treatments or interventions for chronic inflammation. However, currently, there is no method to determine the origin or type of bacterial LPS. Given that it is identifiable and classifiable, circulating bacterial DNA may help elucidate the origins and types of bacterial components found in circulation.

High-fat diets trigger metabolic endotoxemia, defined as an increase in plasma LPS, in human and animal models[8]. This surge of bacterial LPS in the peripheral blood likely elicits the observed postprandial increases in leukocyte count and activation. Several recent studies in patients with cardiovascular disease, as well as healthy participants, have reported increased inflammation markers derived from both the innate and adaptive immune systems in response to a high-fat diet[9-12].

Intestinal inflammation and altered intestinal microbial profiles are thought to play key roles in lipid metabolism and the development of chronic, systemic low-grade

inflammation[13]. Moreover, there is evidence of an imbalance in intestinal microbial profiles and disturbance of the primary intestinal defense system in patients with T1D[14,i,15].

Thus, there is undoubtedly a need for data regarding the sources and effects of circulating microbial remnants in patients with T1D.

[i] However, this does not rule out the possibility that locally elevated LPS (e.g. in the peripheral tissues) might be a contributing factor to postprandial inflammation.

2 Review of the literature

2.1 Diabetes

Diabetes mellitus, henceforth referred to as diabetes, is a chronic, progressive metabolic condition characterized by elevated blood glucose. Globally, an estimated 422 million people currently suffer from diabetes[16]. The World Health Organization (WHO) defines diabetes as a fasting plasma glucose concentration at or above 7.0 mM or a plasma glucose concentration of 11.1 mM two hours after the ingestion of a 75-g oral glucose load[17]. In a healthy individual, glucose concentrations are maintained by a balance between insulin secretion and insulin action. In contrast, patients with diabetes show impaired insulin secretion, decreased insulin sensitivity, or both.

One common indicator of glucose control is glycated hemoglobin (HbA1c). HbA1c is formed when glucose binds to the hemoglobin protein[ii], which is found within all red blood cells. HbA1c is measured from the blood and reflects long-term trends in glucose levels. HbA1c is often reported as a percentage, with <6% being a normal value and values above >6.4% being common in diabetes.

In a healthy individual, insulin is secreted in both a constitutive and regulated manner[18,19]. In addition to low levels of constitutive insulin secretion, healthy pancreatic β cells release large bursts of insulin in response to elevated glucose levels. The concentration of glucose determines the magnitude of this insulin burst.

B cell glucose sensitivity is controlled by a multi-step regulatory pathway. Briefly, the protein glucose transporter 2 (GLUT2) internalizes glucose, which is processed and catabolized, generating ATP from ADP. The ATP-ADP ratio in β cells indirectly stimulates the fusion of insulin-containing vesicles with the plasma membrane, resulting in the release of insulin from the cell[18]. Higher glucose concentrations prompt larger numbers of β cells to release insulin, thereby increasing the concentration of circulating insulin. Elevated concentrations of circulating insulin, in turn, increase glucose uptake in peripheral tissues and lead to lower circulating glucose concentrations.

[ii] The glucose-bound protein is then said to be glycated or glycosylated. This is also mentioned in section 2.4.1 when discussing AGEs.

2.2 Classification of Diabetes

Diabetes is conventionally divided into two subgroups: insulin-dependent type 1 and non-insulin-dependent type 2 diabetes. Type 1 diabetes (T1D) is characterized by autoimmune destruction of the insulin-producing islet β cells and generally manifests itself early in life. The symptoms of T1D include fatigue, muscle loss, increased thirst and urination, blurred vision, erectile dysfunction, and pain or numbness in the hands and feet (http://www.nhs.uk). T1D may be accompanied by diabetic ketoacidosis, a life-threatening condition characterized by high levels of blood acids called ketones, which is triggered by insulin insufficiency.

The more common type 2 diabetes (T2D) is a late-onset disease characterized by hyperglycemia caused by impaired insulin secretion, insulin resistance and increased glucose output by the liver[20]. T2D is generally diagnosed later in life and is often associated with obesity and metabolic syndrome (MetS). However, in recent years an increase in T2D in children and adolescents has been reported[21]. The symptoms of T2D include fatigue, hunger, increased thirst and urination, blurred vision, or pain or numbness in the hands and feet[22].

Three less common forms of diabetes are gestational diabetes, latent autoimmune diabetes in adults (LADA) and maturity-onset diabetes of the young (MODY). Gestational diabetes is defined as any degree of glucose intolerance with onset or first recognition during pregnancy[23]. Similar to T1D, gestational diabetes is diagnosed using a glucose tolerance test; however, the cutoffs vary greatly[24]. Gestational diabetes often resolves itself in the early postnatal period. LADA presents the same clinical symptoms as T2D but also is characterized by islet autoimmunity[25]. LADA is generally diagnosed in young adults, but many LADA cases may be misclassified (e.g., as T1D or T2D) due to the application of inconsistent diagnostic criteria. MODY is an autosomal dominant condition with significant genetic heterogeneity[26]. Although there are six MODY subtypes, the three most common genes that are mutated in MODY patients are *GCK*, *HNF1A* and *HNF4A*[27]. An early-onset disease, MODY generally does not result in patient insulin dependency, and the disease rarely leads to vascular complications[28].

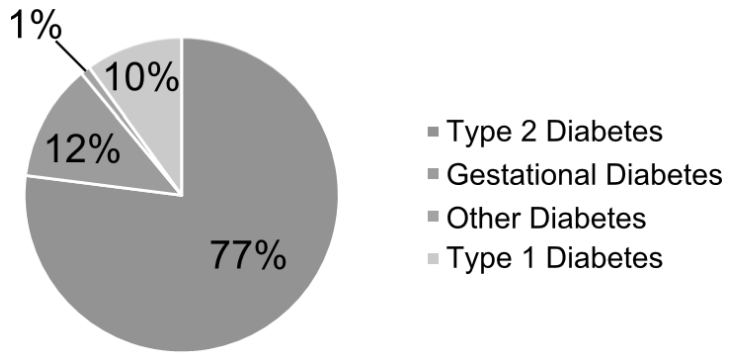

Figure 1 The relative prevalence of types of diabetes.

2.3 Type 1 Diabetes

Type 1 diabetes (T1D) is characterized by the autoimmune destruction of insulin-producing islet β cells early in life.

2.3.1 Epidemiology

Globally, the incidence of T1D appears to be increasing at an alarming rate while the age of onset is decreasing[29,30]. Finland has the highest incidence of type 1 diabetes worldwide with an incidence of over 60 per 100,000 people[1]. In recent years, the incidence of T1D in Finland has increased significantly faster than anticipated; however, the latest data suggest this increase in incidence is leveling off[1].

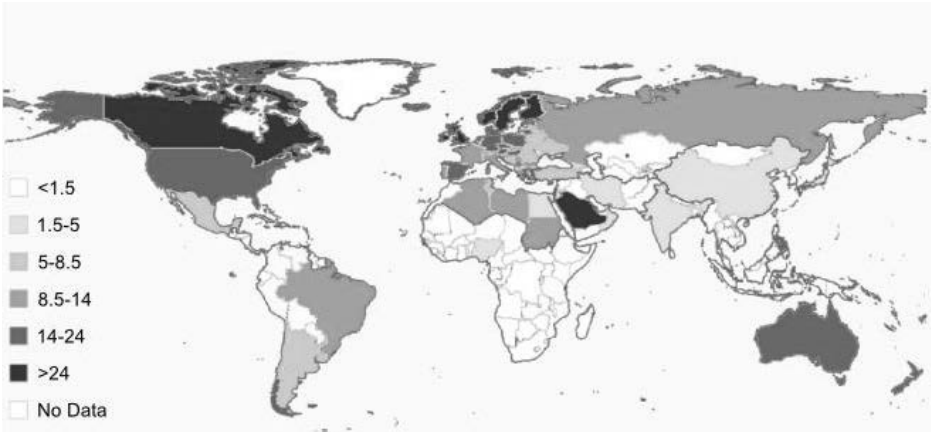

Figure 2 Number of new cases of type 1 diabetes in children 0-14 years old per 100,000 people 2015 [31].

Contradicting the theory of increasing incidence, studies from Sweden and Belgium have reported that the cumulative incidence of T1D has, in fact, remained steady since the late 1980s[32,33]. Hence, ostensible increases in incidence may be attributable to the diagnosis of earlier onset cases[34]. The concurrent decrease in incidence in older age groups may offset the increase in early childhood cases, a situation referred to as 'spring harvest'. Much of the epidemiological data on T1D has been collected in children under the age of 14, so the picture is far from clear at this point. However, in those studies that have included older cohorts, the shift to earlier diagnosis does not fully explain the reported increases in incidence. Studies from Finland, Italy and the UK have also demonstrated increased incidence in older age groups (under 40)[30,35]. Therefore, the epidemiological evidence points to an environmental component in the development of T1D. This has led researchers to investigate a myriad of influencing factors, from diet and lifestyle to infections. A better understanding of the factors that trigger T1D is integral to the development of future treatments.

2.3.2 Pathogenesis

T1D is characterized by polygenic inheritance with generally low penetrance[36]. Mutations in the HLA region of chromosome 6p21 are perhaps the most well-characterized group of genetic risk markers, and approximately 30% of European T1D patients are heterozygous[37]. However, recent studies have reported a decrease in high-risk HLA genotype frequency among more recently diagnosed T1D patients. Combined with the rising incidence of T1D, these data suggest changing genetic and environmental factors are contributing to an increase in incidence among children who lack the high-risk genotypes[38].

Moreover, the disease prevalence within populations carrying genetic risk markers further implicates an outside force in triggering the disease[36]. To date, there have been a myriad of proposals regarding the precipitating event(s) in T1D[39-41]. A viral, bacterial, chemical or dietary factor, alone or in cooperation with other factors, may contribute to the initiation of autoimmunity in T1D patients.

A detailed enumeration and explanation of the early events during the pathogenesis of T1D is outside the scope of this thesis. However, a brief glimpse of the early etiological processes highlights the key roles of inflammation and aberrant cellular

responses. These aberrant responses may also be related to the later development of diabetic kidney disease.

Figure 3 (below) shows the theoretical steps in the development of autoimmune diabetes. Briefly, the initiating event triggers inflammation[iii] (B1), causing an effector T cell[iv] response to be favored over that of regulatory T cells (Tregs) (C1). Concurrently, within the pancreatic β cells, interferon α (IFN-α) (B3) is upregulated followed by MHC class I proteins (C3). Autoreactive CD8 T cells recognize the proteins being presented by the MHC proteins and thus kill the β cells (C3). This releases β cell antigens that are then picked up by antigen presenting cells (APCs) (C3) and brought to the lymph node (C2), where they stimulate CD8 T cell proliferation (D2) and the production of insulin autoantibodies (D1). The autoreactive T cells then migrate to the pancreas (D3), where they secrete perforin, TNF-α and IFN-γ to continue and enhance the autoimmune assault. B cell destruction releases new proteins into circulation, priming subsets of CD4 and CD8 T cells that are specific to new epitopes. This is termed epitope spreading, and it results in T cells specific for new β cell proteins (E2). The secondary assault on β cells is then specific for a broader range of proteins and is, therefore, more severe than the first. The increased inflammation also appears to trigger the proliferation of β cells, while Tregs may occasionally slow the autoimmune destruction of β cells, resulting in a constant fluctuation in the patient's overall β cell mass or function (F3/yellow line). Clinical diabetes is generally diagnosed when the β cells are unable to produce sufficient insulin to prevent hyperglycemia. The 'honeymoon phase' (below F3) is a generally short period soon after diagnosis when the β cells are able to produce sufficient amounts of insulin.

[iii] For a description of this information, please see section 2.7.
[iv] For a brief explanation of the functions of different T cell types, please see section 2.6.

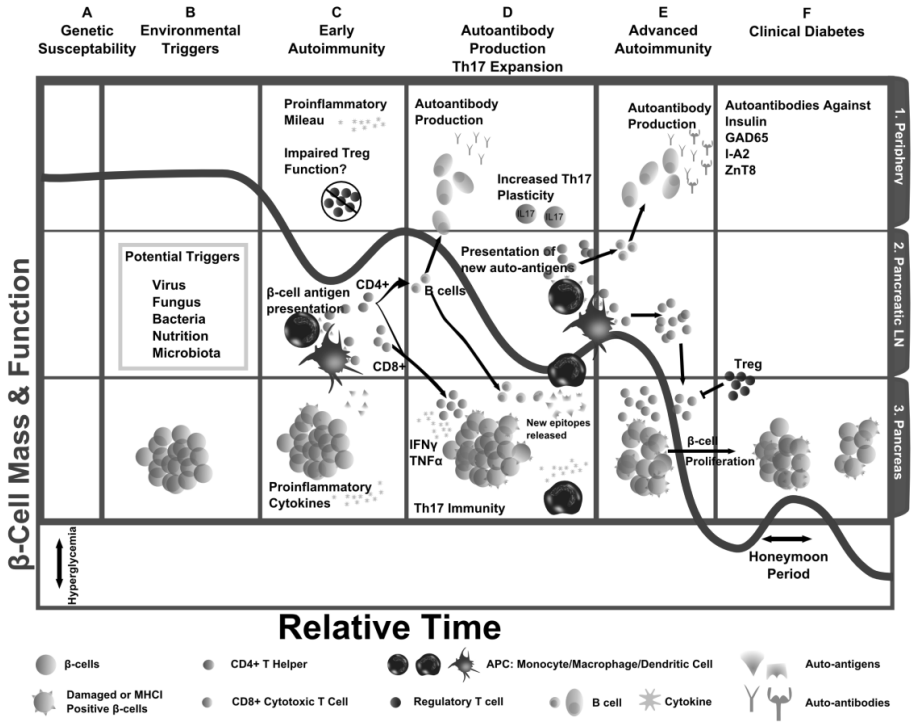

Figure 3 The proposed steps in the etiology of type 1 diabetes. Adapted from[42].

2.3.3 Healthcare Expenditures

As the incidence increases and the age of onset decreases, the costs related to diabetes care will increase. International data on health care expenditures specifically associated with T1D is scarce. Health care costs due to all diagnosed forms of diabetes account for approximately 12% of total global healthcare expenditures[43]. According to the International Diabetes Federation, individual nations generally allocate between 5% and 18% of total healthcare costs to the treatment of diabetes. This includes estimated costs borne by individuals and by the government. There is also evidence that the annual cost of T1D is significantly higher than that of T2D on a per patient basis[44]. Importantly, the development of diabetic complications doubles the cost of treatment[45].

As one of the leading causes of death worldwide, the human cost of diabetes is substantial. There are an estimated 1.5 million deaths related to elevated glucose levels, and this number is expected to double by 2030[17]. It may be possible to stem the increasing rates of morbidity and mortality related to diabetes by improving

treatment for all types of diabetes, particularly aiming to prevent the development of diabetic complications.

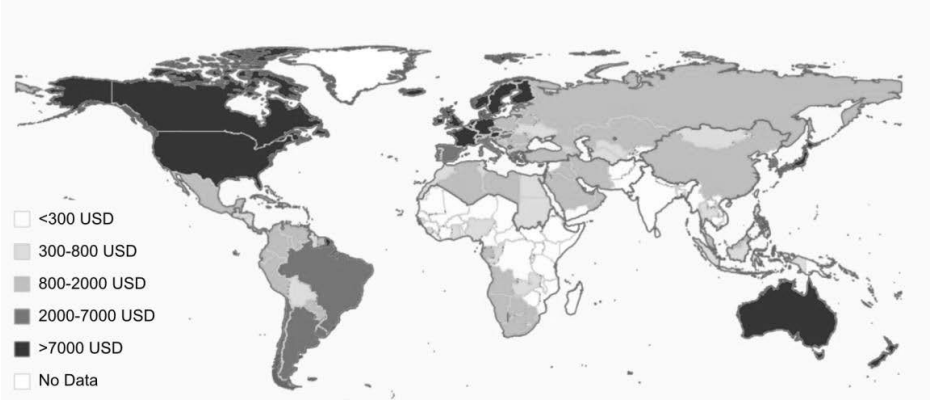

Figure 4 Annual healthcare expenditure per patient with diabetes for 2015, in USD[31].

2.4 Diabetic complications

Broadly speaking, diabetic complications can be separated into two categories: macrovascular[v] and microvascular[vi,46]. Together, these vascular diseases represent the primary cause of morbidity and mortality in patients with T1D. Microvascular and macrovascular complications are frequently observed in T1D patients with long disease durations and metabolic disease[47].

While the precise pathogenesis of diabetic complications is not yet fully understood, morbidity and mortality due to cardiovascular disease, atherosclerosis and nephropathy have been associated with elevated levels of inflammatory markers[3-5]. Moreover, there is growing evidence that the pathogenesis of diabetic complications is also associated with bacterial infections (further discussion below)[7].

Hyperglycemia may play an integral role in immune system weakening and the progression of vascular complications in diabetic patients[46]. This hypothesis is supported by studies showing that hyperglycemia is associated with increased complications and mortality in both patients with T1D and patients with critical illnesses other than diabetes[48,49].

[v] e.g. Coronary artery disease, peripheral arterial disease, stroke.
[vi] e.g. Diabetic nephropathy, neuropathy, retinopathy.

2.4.1 Microvascular Complications

Microvascular complications of diabetes include diseases such as nephropathy, retinopathy and neuropathy. This thesis focuses on diabetic nephropathy and to a lesser extent diabetic retinopathy.

2.4.1.1 Diabetic Nephropathy

Diabetic nephropathy is a degenerative condition characterized by the progressive loss of renal (kidney) function[50]. An estimated one-third of T1D patients develop renal disease 15-20 years after disease onset[2].

The function of the kidney is to regulate the balance of electrolytes, maintain pH homeostasis and filter organic metabolic waste products such as phosphates, sulfates and nitrogen compounds. The basic functional subunit of the kidney is the nephron, which filters the blood and reabsorbs the water, electrolytes and other factors needed to maintain homeostasis. The waste products collected by the nephron are eventually excreted in the urine.

While the healthy kidney filters out metabolic waste[vii], damaged kidneys aberrantly permit beneficial proteins such as serum albumin to leak into circulation. Damaged kidneys are also less efficient at filtering metabolic waste, leading to elevated levels of waste in circulation. If left unchecked, these elevated concentrations of metabolic waste lead to toxicity.

The glomerulus is a nephron subunit that represents the first stage of the filtration process in the kidneys. The glomerulus is partially encapsulated by the podocyte, which works with the glomerulus to form a filtration barrier. In diabetic kidney disease, glomerular basement membranes are thickened, gradually lose their permeability and are subject to leukocyte adhesion[51,52]. Moreover, in diabetic kidney disease, podocytes are less numerous and show signs of structural injury and decreased functionality, leading to the leakage of serum proteins such as albumin into the urine[53,54]. Urine albumin levels are therefore useful for assessing levels of kidney damage.

[vii] Such as creatinine.

A damaged kidney in the context of diabetes is termed diabetic nephropathy. Definitions of diabetic nephropathy are presented in Table 1. Diabetic nephropathy is among the most serious diabetic complications and may lead to decreased quality of life and increased risk of early mortality. Furthermore, diabetic nephropathy is the strongest risk factor for cardiovascular outcomes and is associated with metabolic syndrome[55].

In later stages of diabetic nephropathy, dialysis and kidney transplant may be required.

Nephropathy status	Definition
Normal AER	UAER<30 mg/24 h or UAER<20 µg/min*
Microalbuminuria	30<UAER≥300 mg/24 h or 20<UAER≥200 µg/min*
Macroalbuminuria	UAER≥300 mg/24 h or UAER≥200 µg/min*
End Stage Renal Disease	Dialysis or transplant required

Table 1 Definitions of nephropathy status. UAER refers to the urinary albumin excretion rate determined by a timed urine collection. *Denotes cutoffs for overnight collection.

Chronic hyperglycemia is a major risk factor that is integral to the development of diabetic nephropathy. One underlying mechanism is the formation and accumulation of advanced glycosylated end products (AGES)[56]. AGES are proteins or lipids that have become bound to a sugar molecule and are increased in patients with T1D with a further increase in patients with diabetic nephropathy[52,57]. AGEs may then be deposited on the vascular wall or in the glomerulus, thereby causing thickening of the basement membrane.

AGEs also modulate inflammation and immune cell function via the receptors RAGE and AGE-R2[58-60]. AGEs may, therefore, contribute to podocyte damage through their proinflammatory effects[viii].

While there is only limited knowledge regarding the pathophysiological mechanisms underlying podocyte damage and loss in diabetic nephropathy, recent evidence suggests inflammation plays a role[61]. Indeed, in two recent studies, we reported an association between diabetic nephropathy and elevated levels of inflammatory markers and bacterial endotoxins[6,7].

2.4.1.2 Diabetic Retinopathy

With approximately 360,000 new cases per year in the US alone, diabetic retinopathy (proliferative retinopathy/diabetic macular edema) may be the most prevalent diabetic microvascular complication and frequently precedes diabetic nephropathy[46]. Indeed, one large study found the 25-year cumulative incidence of macular edema to be 29% in patients with T1D[62]. The incidences of diabetic proliferative retinopathy and diabetic macular edema are roughly equivalent[63]. However, proliferative retinopathy is the most severe form of retinopathy and is the most common reason for laser treatment in patients with T1D[64]. Similar to diabetic nephropathy, hyperglycemia is associated with the development of diabetic retinopathy. It is therefore unsurprising that the incidence of retinopathy is positively associated with the development of nephropathy. Diagnosis of retinopathy is based on direct inspection of the retina via fundus photography or direct ophthalmoscopy[65]. While monitoring and clinical management of risk factors are the preferred treatment option for retinopathy, proliferative retinopathy may be treated using methods such as photocoagulation and vitrectomy[66].

[viii] AGEs induce NF-κB-based inflammation both through their receptors as well as directly through TLR4[58-60]. For more information on the relevance of this inflammation in diabetic nephropathy, please refer to sections 2.8 and 2.11.

2.4.2 Macrovascular Complications

Macrovascular complications include diseases such as angina, myocardial infarction, angioplasty, coronary artery bypass graft, stroke, claudication, or peripheral bypass[67]. While macrovascular complications are not discussed herein, hyperglycemia-mediated inflammation is also implicated in their development[68].

2.5 Metabolic syndrome

The first formal definition and list of diagnostic criteria for metabolic syndrome (MetS) were given in 1998 by the World Health Organization[69]. This statement implicated several metabolic and underlying risk factors in the development of the condition and identified certain combinations of risk factors as characteristic of the disease, which were intended for use as diagnostic criteria. These diagnostic criteria have since been challenged and modified, leading to some confusion regarding how to diagnose patients with the syndrome[70,71]. Despite disagreement over the diagnostic criteria, it is generally acceptable to apply the term MetS to the condition characterized by the presence of multiple metabolic risk factors for cardiovascular disease and diabetes[72]. Atherogenic dyslipidemia, elevated blood pressure, elevated glucose, a proinflammatory state, and a prothrombotic state are the five primary metabolic risk factors considered when diagnosing MetS [71]. In addition to metabolic risk factors, there are many underlying risk factors that contribute to MetS, including obesity, physical inactivity, blood pressure, atherogenic diet, primary insulin resistance, advancing age and hormonal factors. The International Diabetes Federation Task Force on Epidemiology and Prevention, the National Heart, Lung, and Blood Institute, the American Heart Association, the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity released a joint statement on the definition of MetS in 2009, requiring that patients meet three of the five criteria presented in Table 2.

As shown in recent analyses of large prospective studies, MetS is an independent risk factor for the development of cardiovascular events and cardiovascular and diabetes-related mortality, even after adjustment for traditional risk factors[55].

Joint Statement Criteria for Metabolic Syndrome
Elevated BP (≥130/85 mm Hg or on drug therapy)
Plasma TG ≥150 mg/dL, 1.7 mmol/L
HDL < 40 mg/dL (1.0 mmol/L) (M), < 50 mg/dL (1.3 mmol/L) (F)
Fasting glucose ≥ 100 mg/L
Elevated waist circumference (varies by country)

Table 2 Definition of metabolic syndrome. BP is blood pressure. TG is circulating triglycerides. HDL is circulating high-density lipoproteins. (M) and (F) refer to criteria for men and women, respectively. Based on the Joint Statement[73].

2.6 The Innate and Adaptive Immune System

In humans, there are two classes of the immune response to invading pathogens: innate (natural, or *a priori*) and adaptive (or acquired) immunity. The adaptive immune response is primarily mediated by B and T cell lymphocytes and characterized by lymphocyte recognition of an immunogenic epitope followed by cellular signaling and proliferation. Depending on the baseline prevalence of lymphocytes that are reactive towards an epitope, as well as antibody affinity, a full response may take weeks to mount.

In contrast, the innate immune response is an evolutionarily conserved, ancient mechanism that facilitates a rapid response to components of invading pathogens, which are generally referred to as pathogen-associated molecular patterns (PAMPs) (see Table 3 in section 2.8)[ix]. Unlike the adaptive immune response, the innate immune response is based on soluble and cellular pattern recognition receptors (PRRs)[x] expressed throughout the organism. Upon ligation of a PAMP, innate immune receptors rapidly mount a first-line response against the invading pathogen.

2.7 Inflammation in Diabetes

According to Buchman *et al*., "inflammation is a cooperative response involving multiple cell types, orchestrated both locally and remotely, and affecting the host at multiple levels of resolution (from organism to gene expression)" [74].

[ix] For example, lipopolysaccharide.
[x] For example, Toll-like receptors.

When antigen presenting cells come into contact with a pathogen or a component thereof, they release a number of cytokines and chemokines. The cytokines and chemokines (further discussion in section 2.11) that are released are called inflammatory markers or mediators and are responsible for the vasodilatation and cellular permeability that manifest as redness, swelling and heat.

Aberrant regulation of the inflammatory process is thought to be the underlying cause of many diseases, including autoimmune disorders, some cancers, allergies, asthma, sepsis, atherosclerosis, and neurodegenerative diseases[75]. Systemic and local inflammation are widely believed to play roles in the pathogenesis and progression of T1D through the aberrant secretion of or reaction to inflammatory markers[76].

There is evidence that pro-inflammatory cytokine secretion is dysregulated in T1D. Two recent studies have demonstrated increased cytokine secretion in response to bacterial lipopolysaccharide (LPS) stimulation in patients with T1D compared to healthy participants[77,78].

Even without experimental stimulation, T1D patients exhibit increased levels of inflammatory cytokines compared to healthy controls[79,80]. In particular, IFN- α, interleukin-1β (IL-1β), IFN- γ and CXCL-10 levels are elevated in recent-onset patients. In patients with a long history of T1D, increased levels of inflammatory markers positively correlate with the progression of diabetic complications such as cardiovascular disease, atherosclerosis and nephropathy, as well as all-cause mortality[3-5].

Given that researchers have reported increased stimulation capacity in human immune cells and increased concentrations of circulating ligands that are able to elicit inflammatory responses, there appear to be myriad causes of elevated inflammation in T1D patients. However, a better understanding of the causes and consequences of increased inflammation may help researchers develop treatments to forestall the development of diabetic complications.

2.8 Bacterial Lipopolysaccharides and Toll-like Receptor 4

Lipid-soluble lipopolysaccharide (LPS, also known as endotoxin) is a PAMP that is often used to model the effects of bacterial infection. LPS is a component of the outer membrane of Gram-negative bacteria[81]. Figure 5 illustrates the composition of the LPS

molecule and its interactions with other components of the bacterial cell envelope. LPS is composed of a conserved, hydrophobic lipid A domain attached to sugar chains of variable length[82]. The sugar chain is subdivided into three domains. Two core oligosaccharide domains are directly attached to the lipid A domain, followed by the distal O antigen domain (Figure 5).

Lipid A is produced constitutively and is highly conserved between bacterial strains[83]. As an integral component of the outer membrane of Gram-negative bacteria, Lipid A is essential for the growth and maintenance of bacterial colonies. The extremely high level of homology[xi] of the bacterial LPS core makes it a prime target for the innate immune system.

LPS is commonly measured using the limulus amebocyte lysate (LAL) assay[84]. This assay measures LPS activity and approximately translates into biologically active, free LPS levels. The LAL assay utilizes a slightly modified amebocyte extract called *Limulus polyphemus* from the North American horseshoe crab. The introduction of LPS to this mixture initiates a biochemical cascade ending in cleavage of the chromophore p-nitroaniline and the development of a yellow color.

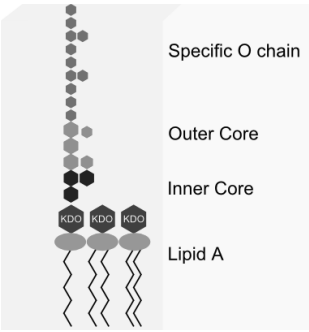

Figure 5 The structure of LPS. Based on[83].

The inflammatory and immunostimulatory effects of LPS have been well-characterized in humans[85-88]. Injected LPS has been applied in humans as a model for the biological response to infection[85]. In animal models, LPS has also been used to

[xi] While the core of LPS is rather homogenous, LPS molecules are highly heterogeneous, particularly with respect to size.

induce kidney failure[89]. Similarly, endogenous serum LPS activity levels are associated with markers of inflammation, as well as the advancement of diabetic nephropathy in T1D[6,7].

LPS triggers inflammation and immune responses via the interaction between the lipid A region of LPS and toll-like receptor (TLR)-4, which is located on the surface of immune and epithelial cells. TLRs are a well-characterized class of transmembrane proteins that detect components of infectious agents and activate host innate and adaptive immune systems in response to pathogen binding (Table 3).

PRR	PAMP
TLR1	Triacyl lipopeptide, with TLR2
TLR2	Bacterial peptidoglycan (PGN)
TLR3	Double-stranded RNA (dsRNA)
TLR4	Lipopolysaccharide (LPS)
TLR5	Flagellin
TLR6	Lipoteichoic acid, diacyl lipoproteins, zymosan
TLR7	Single-stranded RNA
TLR8	Single-stranded RNA
TLR9	Unmethylated CpG DNA
TLR10	Diacyl lipopeptide, with TLR2

Table 3 Pattern recognition receptors (PRRs) and their associated pathogen-associated molecular patterns (PAMPs). Based on [90].

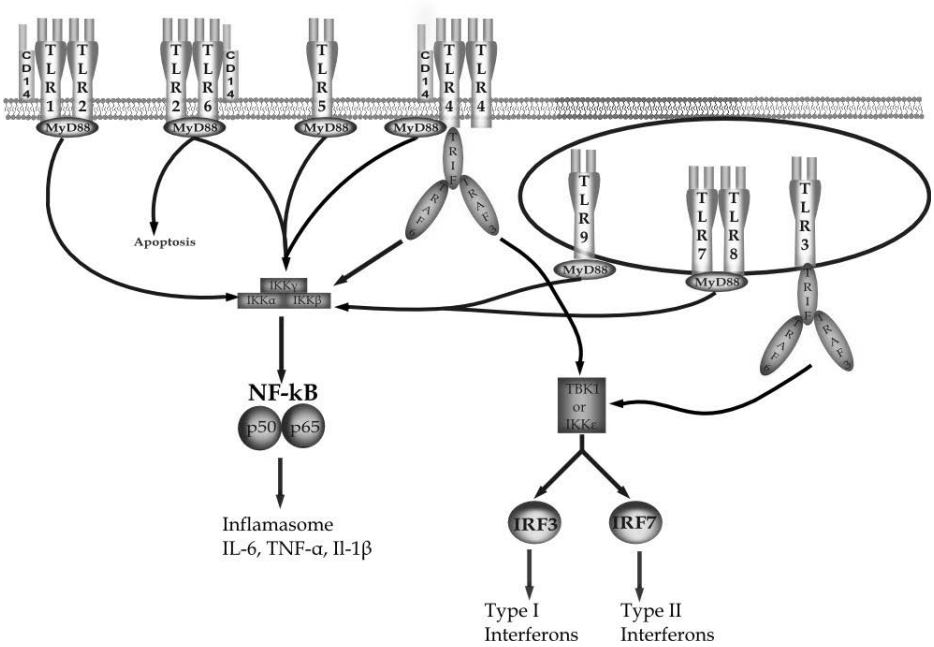

Figure 6 Intracellular signaling pathway for common PRRs. Based on [91].

As shown in Figure 6, the primary intracellular signaling pathway for TLR4 utilizes the MyD88 adaptor protein to trigger the NF-κB inflammatory pathway (discussed further in section 2.10)[92]. TLR4 also triggers immune responses via NF-κB-independent pathways, including the interferon regulatory factor (IRF) pathway. However, the IRF pathway will not be discussed in detail herein.

Recently, it has become increasingly clear that LPS and TLR4 play central roles in the progression of diabetic kidney disease. In humans, a large prospective study showed that LPS activity levels (measured using the LAL assay) predict deteriorating renal function[7]. Patients whose renal function deteriorated had significantly higher LPS activity levels at baseline when compared to those patients whose renal function did not deteriorate. Although the origin of the LPS was not known, patients with T1D suffer from an increased rate of infections[93]. Significantly, the relative prevalence of gastrointestinal infections in patients with T1D was more than double the prevalence observed in healthy controls. However, there is no validated method for determining the origin of LPS in biological samples.

Kidney biopsies from patients with T1D and diabetic nephropathy demonstrate increased TLR4 expression[94]. Moreover, there appears to be a molecular link between TLR4 activation and injury in human kidneys. Indeed, according to recent *in vitro* reports, LPS activation of TLR4, as well as diabetic serum, causes podocyte apoptosis[61]. This is particularly intriguing given that LPS-induced apoptosis is blocked and partially reversed by TLR4 blockade. Furthermore, TLR4 knockout reduces renal inflammation and protects against kidney damage[95]. Strengthening the case that LPS is a major apoptosis-triggering factor in diabetic serum, sera from non-diabetic patients with high LPS activity levels were found to induce apoptosis in kidney cells[96]. This pro-apoptotic activity was abrogated upon incubation with polymyxin B, a compound that sequesters LPS from circulation and therefore inhibits TLR4 binding.

In addition to increased LPS activity levels, there have been numerous reports of increased TLR4 expression in T1D monocytes[78,97,98]. Moreover, this reported increase in TLR4 coincides with increased LPS activity in circulation[97]. Increased LPS levels in combination with increased TLR expression may partially explain the elevated levels of inflammatory markers in circulation in patients with T1D[98,76].

Thus, LPS binding to TLR4 exerts a direct apoptotic effect on kidney cells *in vitro* and is likely a driving force underlying the development of diabetic kidney disease.

2.9 Bacterial DNA and Toll-Like Receptor 9

Another common substance that is used to model bacterial infection *in vitro* is bacterial DNA, which is recognized by TLR9 (Table 3). Bacterial DNA contains a high frequency of unmethylated CpG motifs, which TLR9 recognizes[99]. In contrast, mammalian DNA contains a much lower frequency of CpG motifs, and these are generally methylated. Upon the ligation of unmethylated CpG DNA, TLR9 activates the NF-κB and IRF pathways, similar to LPS ligation of TLR4.

Although the precise half-life of bacterial DNA in circulation is not known, studies have shown that other forms of DNA injected into humans and animals are rapidly cleared from circulation[100,101]. However, it is unlikely that bacterial DNA enters circulation as free DNA; rather, it enters through intact or damaged bacteria. Indeed, bacterial DNA has been identified in certain body compartments up to 7 years after infection[102].

Bacterial DNA in circulation correlates with inflammation, oxidative stress and the presence of bacterial translocation into circulation in the absence of overt infection[103-105]. However, the origin of circulating LPS and bacterial DNA is not known, although there is evidence implicating the oral cavity and the gut as potential sources of bacterial components in circulation. The human intestine is home to a large bacterial population. One early study investigating the diversity of the human microbiome sequenced bacterial ribosomal DNA and created a phylogenetic tree using a 99% similarity cutoff[106]. This phylogenetic tree identified 395 phylotypes, 62% of which were novel and 80% of which represented sequences that had not previously been cultivated at that time. While there is a high degree of diversity in the intestinal flora, the most prevalent bacterial phyla identified were *Firmicutes* and *Bacteroides*. *Proteobacteria*, *Actinobacteria*, *Fusobacter*, and *Verrucomicrobia* were also present but to a noticeably lesser degree. Along with the gut, the oral cavity represents one of the most likely sources of bacterial remnants found in circulation. Periodontitis, an inflammatory gum disease, is more prevalent in patients with T1D than the general population[107]. Studies in patients with periodontitis have shown that mastication increases the levels of circulating endotoxins, suggesting bacterial components enter the bloodstream through infected gums[108].

2.10 NF-κB

Cells employ a cascade of protein interactions to effectively translate PRR ligation into cellular responses. The NF-κB pathway is a major signal transduction pathway initiated upon TLR4 binding to its ligand LPS. NF-κB refers to a homologous, highly conserved group of DNA-binding proteins within the Rel/dorsal family[109]. These cytoplasmic proteins are almost universally expressed and involved in cell stress responses. Although NF-κB is involved in cell survival, proliferation and neural plasticity, the focus herein is its effects on inflammation.

NF-κB is integral to the development of diabetes. NF-κB knockout mice are resistant to streptozotocin-induced diabetes[110]. Furthermore, NF-κB inhibition protects pancreatic islet cells from cytokine-induced apoptosis[111]. Additionally, NF-κB is involved in the progression of diabetic complications such as diabetic kidney disease[96].

Therefore, NF-κB may be a promising therapeutic target for protection against diabetogenesis and the progression of diabetic complications.

2.11 Cytokines

When investigating the roles of cytokines and chemokines in the pathophysiological processes underlying diseases, it is important to note that these inflammatory mediators often trigger a wide array of cellular responses. In addition to the regulation of immune responses, cytokines stimulate proliferation, differentiation and apoptosis. They also influence cell-cell interactions, modulate the adaptive immune process and induce or suppress the production of or responsiveness to other cytokines.

The effects of cytokines on renal injury were first described in 1991 when Hasegawa[112] reported the ability of diabetic kidney glomerular basement membranes and mesangial matrix from mice to induce TNF-α and IL-1β secretion in cultured macrophages. Shortly thereafter, Sekizuka *et al.* reported elevated IL-6 concentrations in patients with diabetic nephropathy[113].

These early studies opened the door to a number of studies investigating the effects of cytokines on diabetic nephropathy. It is now well established that elevated cytokine concentrations are associated with the progression of diabetic complications[3-5]. Specifically, markers of inflammation such as IL-18, IL-6, IL-1β, CRP, MBL, TNF-α and ICAM-1 are elevated in patients with diabetic nephropathy[5,114-117]. Studies examining the progression of diabetic complications and upstream mediators of inflammation such as LPS-TLR4-NF-κB (see above) suggest these cytokines may be directly linked to the initiation of kidney cell apoptosis and the associated decline in renal function.

Upon exposure to a broad array of cytokines, pancreatic β cells modify their expression of numerous genes, eventually leading to apoptosis. Cardozo *et al.* reported more than 66 genes in rat pancreatic β cells that are differentially expressed upon exposure to cytokines[118,119]. Events during the early pathogenesis of diabetes may also play a role in the eventual progression of diabetic complications.

2.11.1 Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine that has long been known to play a role in insulin resistance[120]. It is produced in a wide array of cells,

including renal cell types and kidney-resident immune cells[121-124]. Furthermore, TNF-α is commonly upregulated in response to TLR4 binding to LPS[125]. TNF-α directly induces renal cell damage and apoptosis[126,127]. Moreover, it initiates a local pro-apoptotic milieu by triggering the production of inflammatory mediators and other proteins involved in immune cell recruitment[128,129].

During earlier stages of diabetic kidney disease, TNF-α induces the early signs of renal dysfunction, renal hypertrophy and hyperfiltration in rats[130,131], which are measured in humans as the albumin excretion rate.

2.11.2 IL-1β

Similar to TNF-α, interleukin (IL)-1β is produced at sites of local inflammation and is a pleiotropic inflammatory mediator. At the genetic level, polymorphisms in the gene encoding IL-1β have been associated with the risk of diabetic kidney disease[132], potentially due to the known effects of IL-1β on renal hemodynamics or vascular endothelial cell permeability[133,134]. Recently, Shahzad *et al.* demonstrated that increased concentrations of IL-1β in circulation proceeded albuminuria and glomerular extracellular matrix accumulation in a murine model of T1D[135]. Furthermore, inhibition of IL-1 receptor signaling reduced albuminuria. Taken together, these data suggest IL-1β up-regulation plays a key role in the development of diabetic nephropathy.

2.11.3 IL-6

In an early study, we showed that one of the primary regulators of inflammation in the human body, IL-6, is associated with progressive diabetic nephropathy[115]. Furthermore, polymorphisms in the *IL6* gene promoter are associated with diabetic kidney disease[136]. Similar to TNF-α and IL-1β, the mechanisms underlying the association between IL-6 and diabetic kidney disease involve a wide array of pathways. In particular, IL-6 is involved in endothelial permeability, the induction of mesangial cell proliferation, and increased fibronectin expression[137].

2.12 Fat-enriched diets

High-fat diets have been shown in human and animal models to trigger metabolic endotoxemia, defined as an increase in plasma LPS[11,138]. Although the mechanism underlying the internalization of LPS has yet to be confirmed, LPS is most likely internalized by the Golgi complex in intestinal epithelial cells, where it is then transported into circulation by chylomicrons that are synthesized in response to the fat load[10,139,140]. Increased chylomicron production triggered in response to a high-fat diet is proposed to increase the transport of LPS from the gut into circulation.

This surge in bacterial LPS in the peripheral blood likely also elicits the observed postprandial increase in leukocyte count and activation[141-143]. Several recent studies in patients with cardiovascular disease, as well as healthy participants, have reported increases in inflammation markers from both the innate and adaptive immune systems in response to a high-fat diet[9-12]. Additionally, a high-fat diet has been shown to increase LPS sensitivity and trigger the release of inflammatory markers, notably IL-6 and TNF-α, in a murine model[143,144].

2.13 Intestinal Inflammation in T1D

Intestinal inflammation and altered intestinal microbial profiles are thought to play key roles in lipid metabolism and the development of chronic, systemic low-grade inflammation[13]. Previous studies have revealed systemic inflammation without evidence of elevated leukocyte cytokine production in response to a series of high-fat meals (Study II). We observed no changes in cytokine secretion or LPS sensitivity in immune cells; therefore, commonly observed postprandial inflammation likely originates in peripheral tissues and is not triggered by systemic postprandial LPS[xii]. Furthermore, patients with T1D exhibit impaired triglyceride metabolism in response to a high-fat meal[14]. Taken together, these observations suggest an imbalance in intestinal microbial profiles and concomitant disturbance in the primary defense system in the intestines.

[xii] However, this does not exclude the possibility that locally elevated LPS (e.g. in the peripheral tissues) might be a contributing factor to postprandial inflammation.

Evidence has suggested the presence of subclinical inflammation in the intestines of patients with T1D[15]. Indeed, T1D shares several genetic and immunological components with inflammatory bowel disease (IBD)[145-147]. Furthermore, these diseases share an increased risk of cardiovascular complications[148-150]. Previous studies have shown an increased prevalence of IBD in patients with T1D but not an increase in T1D in patients with IBD[151,152]. These studies also found similar HbA1c levels in T1D and T1D + IBD populations, suggesting the increased prevalence is not secondary to poor glycemic control.

2.14 Alkaline phosphatases

Alkaline phosphatases (APs) are a group of enzymes derived from the liver, bone, placenta and intestine that are found in circulation and feces[153]. The intestinal isoform of AP (IAP) plays a key role in the maintenance of intestinal homeostasis[154,155]. IAP, a protein produced in the enterocyte brush border, is able to hydrolyze monophosphate esters[156]. IAP detoxifies LPS through dephosphorylation of the lipid A moiety [154,155]. Triglycerides are transported into circulation through chylomicrons, which are known to incorporate IAP and LPS. Therefore, both circulating IAP and LPS increase upon the ingestion of fatty meals[157]. However, we recently showed that this postprandial increase in LPS activity levels is rather modest and not seen in patients with T1D[14]. Furthermore, in an earlier study, we found no association between circulating LPS and markers of inflammation[14]. By contrast, injected LPS rapidly upregulates inflammatory cytokines.

IAP is critical for the regulation of fatty acid absorption, and IAP deficiency leads to the development of metabolic syndrome in mice[155,159]. Furthermore, IAP insufficiency has been observed in intestinal diseases related to T1D, such as inflammatory bowel disease and celiac disease[160-162]. However, these studies did not control for blood type or secretor phenotype, which is a genetic component that strongly affects IAP activity[157]. IAP supplementation may nevertheless represent one potential therapeutic strategy for inflammatory bowel disease and metabolic syndrome[163,164].

Although IAP expression has a strong genetic component, it is also influenced by diet and participates in bidirectional regulation with the gut microbiota, i.e., IAP expression affects microbial composition, and microbial composition affects IAP expression[155].

One mediator of the IAP-microbiota interaction is the short-chain fatty acid butyrate. Butyrate, along with the short-chain fatty acids acetate and propionate, is produced in the colon by commensal microbiota that ferment complex carbohydrates and plant polysaccharides[165]. While butyrate serves as an energy source for the colonic epithelium, it has also been reported to enhance both IAP expression and activity[166]. Given that butyrate is a microbial fermentation product of dietary components, it is not surprising that diet indirectly regulates the production, secretion and biological activity of IAP[153].

3 Aims of the Study

Based on our prior observations, we hypothesized that bacterial infections play a significant role in the development of diabetic kidney disease. Although a number of previous studies have reported increased morbidity and mortality due to T1D-associated infections, the cause of this increased susceptibility remains unclear. There is evidence of a dysregulated innate immune system in T1D; however, a concrete link between this dysregulation and differential inflammation and susceptibility to infections has not been established. The primary goal of the present study was to identify biological factors that modulate bacteria-mediated inflammation in patients with T1D.

The specific aims of studies I-III were as follows:

I. Determine the origin of bacterial DNA in circulation and investigate correlations with markers for immune response and inflammation.
II. Determine the acute effects of high-fat meals on inflammation and the inflammatory response of antigen presenting cells in the context of T1D.
III. Determine the role of intestinal alkaline phosphatase in intestinal inflammation and immune response.

4 Materials and Methods

4.1 Study Subjects

Adult subjects with and without T1D were investigated in studies I-III (Table 4). Healthy non-diabetic control subjects (NDC) and patients with T1D were selected from the Finnish Diabetic Nephropathy Study (FinnDiane). T1D was defined as the onset of diabetes before the age of 40 years and permanent insulin treatment initiated within 1 year of diagnosis. Normal albumin excretion was defined as AER<20 μg/min or <30 mg/24 h. Macroalbuminuria was defined as AER≥200 μg/min or ≥300 mg/24 h

	T1D	**NDC**	**Macro**
Study I-DNA	14 (8/6)	10 (5/5)	-
Study I-Serum Ab	200 (97/103)	200 (94/106)	-
Study II	11 (6/5)	11 (7/4)	-
Study III	36 (14/22)	41 (20/21)	10 (7/3)

Table 4 Participants in each of the three studies. Numbers represent total numbers of subjects (male/female). NDC: Non-diabetic controls; macro: patients with T1D and macroalbuminuria.

Subjects within the circulating DNA study (Study I)

Participants were recruited by the FinnDiane Study. Study I included 10 non-diabetic control subjects and 14 patients with T1D. Unless otherwise noted, all tests were performed using blood samples taken from these subjects. We hypothesized that individuals with high LPS harbored higher overall bacterial loads. Therefore, all selected subjects belonged to the highest LPS quartile within their group. For serum antibody analysis, additional healthy controls (n = 200) and patients with T1D (n = 200) were randomly selected from the FinnDiane Study.

Subjects within the fat tolerance study (Studies II and III)

Participants were recruited by the FinnDiane Study. Study II included 11 patients with T1D and 11 controls. Study III included 41 non-diabetic controls, 36 patients with type 1 diabetes and normal albumin excretion and 10 patients with type 1 diabetes and macroalbuminuria. The inclusion criteria for studies II and III were 1) age 18-65 years; 2) no use of antibiotics during the past month; and 3) no trips outside the Nordic countries during the past month.

In studies II and III, on the investigation day, participants were given three energy-rich meals (2600 kcal): breakfast (at 8:00 - 965 kcal, 58% of total energy (E%) from fats), lunch (12:00 - 870 kcal, 44 E% fats), and dinner (16:00 - 779 kcal, 46 E% fats). Blood samples were drawn after overnight fasting and every two hours until 18:00 hrs. Urine was collected during the research day. Participants collected two fecal samples before and after the research day, approximately one week apart. The samples were then sent to the study center for further processing. Measured analytes in these two samples strongly correlated. The samples were therefore used as biological replicates.

Participants in Study III completed a three-day food record prior to the day of investigation. Dietary intake was analyzed as previously described [167]. Information on the use of medication was recorded with a standardized questionnaire.

4.2 Laboratory Methods

4.2.1 Measurements from serum, plasma and cell culture supernatant

Analysis of serum LPS activity – Studies I, II and III

In Studies I and III, serum LPS activity was measured kinetically from serum samples using the Limulus amoebocyte lysate assay (LAL, Hycult, Uden, Netherlands). Briefly, color formation at 405 nm was recorded every 2 min for a total of 40 min. We subtracted the minimum absorbance value from the maximum value to correct for interference caused by postprandial triglycerides and hemolysis [168].

Analysis of circulating cytokines – Study II

Serum concentrations of IL-6, TNF-α, IL-1β, IFN-α, IL-10, IFN-γ, IL-12, MIP-1β, IL-1α, IP-10, MIP-1α, IL-8, and MCP-1 were assayed using the FlowCytomix Multiple Analyte System (eBioscience, Vienna, Austria) according to the manufacturer's instructions. Data were analyzed using FlowcytomixPro (eBioscience, Vienna, Austria) and Prism (GraphPad Software, Inc., La Jolla, CA, USA). Standard curves were generated, and concentrations were calculated using Prism.

Intracellular cytokine staining and LPS stimulation – Study II

The production of IL-1β, TNF-α-, and IL-6 was assayed in resting and PBMCs stimulated with exogenous LPS by performing intracellular staining followed by flow cytometry. Briefly, immediately after collection, 200 µl of sodium heparin blood was incubated with or without 100 ng/ml *E. coli* LPS (strain 0111:B4; DIFCO 3122-25-8 Detroit, MI, USA) for 4 h at 37°C. After 1 h, GolgiStop (BD Biosciences, San Jose, CA, USA) was added. Thereafter, blood was mixed with surface marker antibodies to identify monocytes and myeloid dendritic cells (mDCs) (CD14 PE-Cy5.5, CD19 PE-Cy5.5, and CD1c APC) and incubated for 20 min in the dark at room temperature (RT). Red blood cells were then lysed using FACS Lysing Solution (BD Biosciences). White blood cells were pelleted out of solution via centrifugation and washed twice with and fixed with 4% paraformaldehyde in PBS. The cells were then permeabilized using BD Perm/Wash according to the manufacturer's instructions and stained for intracellular cytokines using IL-1β FITC, TNF-α-PE, and IL-6 PE or isotype controls. Staining controls were prepared in the same way using 100 µl of blood.

This stimulation was performed on whole blood; however, LPS activity levels with 100 ng/ml *E. coli* were tested under various in vitro conditions, including cell culture medium supplemented with 10% fetal calf serum. An activity level of 2.01 was observed in 10% serum-supplemented medium (unpublished data). In comparison, healthy adult serum LPS activity levels range between 0.1 and 0.2.

Serum measurements of CRP, AP, lipids, and clinical parameters – studies I, II, and III

High-sensitivity C-reactive protein (hsCRP) was measured in the serum via immunoprecipitation (Thermo Scientific, Vantaa, Finland).

Serum alkaline phosphatase (AP) activities were determined in the Helsinki University Hospital central laboratory and included the quantitation of total, bone, liver, intestinal, and macromolecular alkaline phosphatase activity (HUSLAB, Helsinki, Finland). Serum AP activities were determined using a photometric method. Alkaline phosphatase isoforms were quantified by performing agarose gel electrophoresis.

Blood glucose, urinary albumin, and serum creatinine concentrations were determined by the Laboratory of Helsinki University Central Hospital (HUSLAB, Helsinki, Finland). Kidney status was determined by calculating the urinary albumin excretion rate (AER) and the estimated glomerular filtration rate (eGFR) using the CKD-EPI equation[169]. Serum insulin concentrations were determined with the Wallac AutoDELFIA Insulin kit (PerkinElmer, Turku, Finland) using an automatic analyzer (Wallac 1235 Automatic Immunoassay System, Wallac, Turku, Finland).

Serum triglycerides, total cholesterol, HDL cholesterol, apolipoprotein (apo) A-I, and apoB-100 concentrations were measured using automated enzymatic methods (Konelab analyzer, Thermo Scientific, Vantaa, Finland). Plasma apoB-48 concentrations were measured by ELISA according to the manufacturer's instructions (Shibayagi Co Ltd, Shibukawa, Japan). ApoE concentrations were measured by performing anti-apoE immunoaffinity chromatography[170].

4.2.2 Fecal sample measurements

Analysis of fecal IAP activity – Study III

Fecal IAP activity was measured by performing a colorimetric assay. Briefly, human (fecal) and mouse (cecum, colon) samples were homogenized and centrifuged, and then supernatants were collected to determine fecal IAP activities and total protein concentrations. A standard curve was prepared using serial dilutions of p-nitrophenyl phosphate (pNPP) and a fixed amount of calf intestinal alkaline phosphatase (CIAP) in the final reactions (Sigma). To measure the background signal, fecal extracts and all assay reagents were combined simultaneously into a control well before starting the assay. OD values were determined at 405 nm with the correction wavelength set to 630 nm. Sample activity was calculated using the following formula: IAP activity (U/ml) = A/V/T (A is the amount of pNP generated in µmol, V is the volume of sample in ml,

and T is the reaction time in min). Finally, IAP activities were normalized to the fecal protein concentrations determined using the Lowry method (DC protein assay, BioRad, CA, USA). In the present study, the inter- and intra-assay coefficients of variation for the fecal IAP activity measurements were 13.7% and 2.5%, respectively.

Analysis of fecal short chain fatty acids – Study III

Short chain fatty acids (SCFAs) acetate [C2], propionate [C3], butyrate [C4], valerate [C5] and isovalerate [iC5] were measured with an HP 5890 series gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with an HP-FFAP column (30 m x 0.53 mm; film thickness 1.0 µm) and a flame ionization detector. Briefly, fresh feces were diluted in water and centrifuged. A volume of 23.6 µl of 12 mM isobutyric acid (as an internal standard), 280 µl of 0.36 M $HClO_4$, and 270 µl of 1 M NaOH was added to 50 µl of supernatant. The mixture was lyophilized, and the residue was re-dissolved in a mixture of 400 µl of acetone and 100 µl of 5 M formic acid. After centrifugation at 14000 x g for 5 min at room temperature, 1 µl of supernatant was injected into the gas chromatograph. Standards were incorporated during all runs.

Fecal antibodies – Study III

Total secretory IgA, IgG and IgM antibodies and isotype-specific antibodies against oxidized LDL products (copper oxidized LDL – CuOx-LDL; malondialdehyde acetaldehyde LDL – MAA-LDL) were measured in fecal samples. Briefly, human (fecal) and mouse (cecum, colon) samples were dissolved in ice-cold PBS buffer and centrifuged. Supernatants were supplemented with protease inhibitors. Levels of IgA, IgG and IgM antibody binding to oxidized LDL were determined for the supernatants in a chemiluminescence immunoassay [171]. Two models of oxidized LDL, CuOx-LDL and MAA-LDL, were used in the study [5]. The amounts of bound antibody present were detected with alkaline phosphatase-labeled goat anti-human secondary antibodies for IgA, IgG and IgM (Sigma) using LumiPhos 530 chemiluminescence substrate (Lumigen). Luminescence was detected with a Victor3 multilabel counter, and the results are expressed as relative light units. Total fecal immunoglobulin levels were measured using a capture-sandwich chemiluminescent immunoassay[171]. A standard curve of mixed or purified human IgA, IgG or IgM was used to calculate the concentrations.

Fecal calprotectin – Study III

Fecal calprotectin concentrations were determined by ELISA according to the manufacturer's instructions (Buhlmann, Schönenbuch, Switzerland). Fecal calprotectin concentrations under 50 µg/g were considered normal. Concentrations between 50-200 µg/g implicated increased intestinal neutrophil activity and inflammation. Concentrations above 200 µg/g were indicative of active inflammation and inflammatory bowel disease.

4.2.3 Other measurements from human samples

Determination of the alleles for ABO blood groups and FUT2 by PCR – Study III

To determine *ABO* alleles, the following markers were examined by performing TaqMan real-time PCR: rs8176719, rs8176746 and rs8176747. Briefly, amplification reactions were carried out using 60 ng of template DNA, TaqMan® Universal Master Mix II (Life Technologies, Carlsbad, CA, USA) and primer (600 nM) and probe (100-300 nM) mix (Life Technologies, Carlsbad, CA, USA) in 20-µl reaction volumes. Thermal cycling was performed with the following reaction conditions: denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 3 s and 62°C for 30 s. *FUT2* genotyping was based on the marker rs601338 and analyzed in a similar manner. The distribution of genotype frequencies did not deviate significantly from Hardy–Weinberg equilibrium.

DNA isolation, PCR amplification and cloning – Study I

Nucleic acid extraction, polymerase chain reaction (PCR), and post-PCR analysis were carried out in separate locations to minimize the risk of contamination. Control samples were run in parallel to standardize isolation and transformation procedures and ensure the fidelity of the procedure.

To determine the relative prevalence of bacterial remnants in circulation, DNA was isolated from the sera of 14 high LPS activity patients with T1D and 10 healthy controls. Nucleic acids were isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To improve DNA recovery, 5

µg of Qiagen Carrier (poly-A carrier RNA, Qiagen) was added to each 200-µl serum sample. Ultrapure water was used as a negative control in the extractions.

Previously described primers were used to PCR amplify bacterial 16S rDNA [172]. Adaptor sequences were added to the 5' ends of the 16S primers to facilitate annealing to the pBluescript KS cloning vector.

The thermal cycler (Biometra T Professional Thermocycler, Biometra, Goettingen, Germany) was programmed for an initial step at 98°C for 30 s, followed by 40 thermal cycles of 98°C for 10 s, 55°C for 20 s and 72°C for 60 s. ExoSap-IT (Affymetrix, Santa Clara, CA, USA) was used to remove unincorporated primers and dNTPs according to the manufacturer's recommendations. PCR products were examined in agarose gels stained with ethidium bromide to confirm the product size and the absence of product in the negative control.

After verifying the quality, 5 µl of PCR product was incubated with 2 µl of In-Fusion Cloning Enhancer (Clontech, Mountaincenter View, CA, USA) for 15 min at 37°C, to which we added 90 ng of linearized pBluescript vector (EcoRI/BamHI), 2.8 µl of 5x Fusion Buffer, and 1.2 µl of Fusion Enzyme (Clontech). Ligation buffer was maintained at 37°C for 15-30 min, followed by 15 min at 50°C to stop the reaction.

DNA isolation, PCR amplification and cloning – Study I

Competent DH-5α cells (New England Biolabs, Ipswich, MA, USA) were transformed by incubating with 5 µl of 1:4 diluted ligation buffer. Transformed bacteria were cultured on LB agar plates containing 100 mg/ml ampicillin, 80 µg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), and 1 mM isopropyl thiogalactoside (IPTG) (Sigma–Aldrich, St. Louis, MO, USA). After overnight culture at 37°C, bacteria were collected, and plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmid clones were sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) and BigDye v3.1 according to the manufacturer's instructions. The resulting sequences were analyzed using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences flanking the PCR primers were blasted against microbial genomes using the National Center for Biotechnology Information (NCBI) database. Ten clones from each participant were isolated and analyzed.

Enzyme-linked immunosorbent assays (ELISA) to detect serum antibodies against Pseudomonas species – Study I

An in-house method was carried out to determine serum IgA and IgG antibody levels against *P. aerigunosa* (ATCC27835), *S. maltophilia* (ATCC17671) and *Delftia acidovorans* (ATCC43868), both individually and in combination. An ELISA assay was performed as previously described[173].

4.2.4 Murine experiments

Animals

C57BL/6 mice were purchased from Jackson Laboratories (Sacramento, CA, USA) Animals were maintained in accordance with the guidelines prepared by the Institutional Animal Care and Use Committee at Massachusetts General Hospital, based on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council [Department of Health, Education and Human Services, Publication 85e23 (National Institutes of Health), revised 1985]. All animal protocols were reviewed with approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital.

Animal diets

Liquid Low-fat diet powder (LFD) [D12450HL; 70% kcal from carbohydrate, 20% kcal from protein, 10% kcal from fat] and a liquid high-fat diet powder (HFD) [D12451L; 35% kcal from carbohydrate, 20% kcal from protein, 45% kcal from fat] were purchased from Research Diets (USA).

Oral IAP supplementation in mice

Drinking water and mouse chew diet were removed from all cages prior to beginning the experiments. Mice (n=28) were supplemented with liquid diet feeding bottles containing a suspension made of LFD powder in drinking water to acclimate mice to the liquid diet feeding tubes. One week later, mice were supplemented with liquid diet feeding bottles containing a suspension made of HFD powder +/- different doses

of IAP or its vehicle: HFD + IAP vehicle (n=4), HFD + IAP (1.5, 3.0, 7.5, 15, 30 and 60 U/ml of liquid diet; 4 mice /treatment group) for 11 weeks. The feeding bottles were changed every 12 hours with fresh IAP. In the end of the experiment, IAP treated mice were grouped for subsequent data analysis: low IAP (1.5 and 3.0 U/ml; n=8), medium IAP (7.5 and 15 U/ml; n=8), high IAP (30 and 60 U/ml; n=8).

4.2.5 Statistical methods

Statistical differences between variables were investigated using the Spearman´s Rank correlation test or Pearson's product moment correlation coefficient. Correlations between variables and group differences were determined with Student's t-test, Kruskal-Wallis test, or Mann-Whitney U-test, when appropriate. In the animal experiments, the impact of oral IAP supplementation on caecal and colonic output measures were studied with the Jonckheere-Terpstra trend test. A p-value ≤0.05 was considered significant. Statistical analyses were carried out using SPSS 15.0 (SPSS Inc., Chicago, USA) or the R language and environment package for statistical computing.

4.2.5 Ethical considerations

The research was conducted in accordance with the Declaration of Helsinki. All participants gave informed consent. The research was approved by the appropriate hospital and university ethics committees.

5 Results

5.1 Study I – Systemic exposure to Pseudomonal bacteria: a potential link between type 1 diabetes and chronic inflammation

5.1.1 Relative prevalence of bacterial DNA in serum

To elucidate the origin of bacterial remnants in circulation, bacterial DNA was isolated from the sera of 14 high LPS activity patients with T1D and 10 healthy controls.

With the exception of cyanobacteria, all sequences demonstrated more than 90% homology with the NCBI Microbial Genome database. Bacteria belonging to the genus cyanobacteria showed more than 80% homology with the reference sequences. Among 240 isolated clones, 35 unique bacterial species were identified (Table 5). Only 5 bacterial species were found in both groups. The following bacterial phyla were the most prevalent: proteobacteria (73.8%), firmicutes (13.3%), actinobacteria (5.8%), cyanobacteria (5.0%), and bacteroides (2.1%). The majority (76%) of isolated bacterial clones were Gram-negative. Twenty four percent were Gram-positive.

Over 75% of the isolated bacterial clones had genomic GC content greater than 60%. Approximately 23% were derived from human commensal bacterial flora. Approximately 11% were classified as human pathogens. Fifty-four percent represented environmental bacteria. Eleven percent of the identified species were associated with dairy processing.

D. acidovorans (45%) and *S. maltophilia* (25%) were the most prevalent species. Notably, both belong to the *Pseudomonas* genus. The prevalence of *S. maltophilia* clones was significantly higher in patients with T1D compared to healthy controls (29.3 vs. 18.0%; $p = 0.045$).

Bacterial species	Gram	GC (%)	Type	T1D	Control	ALL
				n **% total**	***n*** **% total**	***n*** **% total**
Acidovorax ebreus	−	67	E	0	1 1.00%	1 0.40%
Bacteroides vulgatus	−	42	H	1 0.70%	0	1 0.40%
Bordetella avium	−	62	E	0	1 1.00%	1 0.40%

Burkholderia phytofirmans	–	63	E	0	1 1.00%	1 0.40%
Cyanothece sp.	–	38	E	0	10 10%	10 4.20%
Delftia acidovorans	–	67	E	62 44%	46 46%	108 45%
Flavobacteriaceae bacterium	–	43	E	3 2.10%	1 1.00%	4 1.70%
L. pneumophila str. Corby	–	39	P	0	1 1.00%	1 0.40%
Lyngbya sp.	–	41	E	1 0.70%	0	1 0.40%
Methylobacterium populi	–	69	E	2 1.40%	0	2 0.80%
Methylibium petroleiphilum	–	69	E	0	1 1.00%	1 0.40%
Nostoc sp.	–	41	E	1 0.70%	0	1 0.40%
Ochrobactrum anthropi	–	56	E	1 0.70%	0	1 0.40%
Stenotrophomonas maltophilia	–	66	E & P	41 29%	18 18%	59 25%
Xanthomonas campestris	–	65	E	0	1 1.00%	1 0.40%
Xanthomonas oryzae pv.*oryzicola*	–	64	E	1 0.70%	0	1 0.40%
Clostridium phytofermentans	+	35	E	0	1 1.00%	1 0.40%
Clostridium sp.	+	50	E	0	2 2.00%	2 0.80%
Clostridium bartlettii	+	29	H	0	1 1.00%	1 0.40%
C. lipophiloflavum	+	65	P	2 1.40%	0	2 0.80%
Enterococcus faecalis	+	38	H	0	2 2.00%	2 0.80%
Exiguobacterium sp.	+	49	E	1 0.70%	0	1 0.40%
Exiguobacterium sibiricum	+	48	E	2 1.40%	1 1.00%	3 1.30%
Geobacillus sp.	+	49	E	4 2.90%	0	4 1.70%

Lactobacillus delbrueckii subsp. *bulgaricus*	+	50	D	1 0.70%	0	1 0.40%
Lactococcus lactis subsp. *cremoris*	+	36	D	3 2.10%	0	3 1.30%
*Lactococcus lactis*subsp. *lactis*	+	35	D	2 1.40%	0	2 0.80%
Micrococcus luteus	+	73	E & H	7 5.00%	0	7 2.90%
Propionibacterium acnes	+	60	H	1 0.70%	4 4.00%	5 2.10%
Staphylococcus haemolyticus	+	33	H	0	1 1.00%	1 0.40%
Staphylococcus warneri	+	33	H	0	1 1.00%	1 0.40%
S. equi subsp. *equi*	+	41	P	1 0.70%	0	1 0.40%
Streptococcus sanguinis	+	43	H	0	3 3.00%	3 1.30%
Streptococcus sp.	+	41	E	0	3 3.00%	3 1.30%
Streptococcus thermophilus	+	39	D	3 2.10%	0	3 1.30%
Total				**140** **100%**	**100** **100%**	**240** **100%**

Table 5 Bacterial clones isolated from human serum. Bacterial classification: E, environmental source; H, human flora; D, dairy products; P, pathogen. Note: percentages may not total 100% due to rounding.

5.1.2 RT-PCR quantification of flagellin DNA in human PBMCs

Real-time PCR was performed to detect flagellin from *S. maltophilia* and a closely related opportunistic pathogen, *P. aeruginosa*, in isolated DNA samples derived from peripheral blood leukocytes. Flagellin PCR was performed on 10 healthy controls and 12 out of 14 patients with T1D included in the serum-derived bacterial DNA study, allowing us to assess the Pseudomonal bacterial load in the human leukocyte DNA fraction.

Primer–probe sets targeting the flagellin genes of *S. maltophilia* and *P. aeruginosa* were tested on DNA extracts from multiple target and off-target bacteria. The probe sets appeared to be specific for the target genes and demonstrated no cross-reactivity.

Standard curves were linear: *S. maltophilia* for 6.4–0.5 × 10^6 copies, and *P. aeruginosa* for 32–2.5 × 10^6 copies. The sensitivity was insufficient to produce a reliable signal in DNA isolated from human serum samples. However, white blood cells are able to carry bacterial DNA; therefore, DNA from circulating white blood cells was used.

All samples assayed by qPCR demonstrated detectable levels of *S. maltophilia* and *P. aeruginosa* DNA. No differences were seen between patients and controls for *S. maltophilia* (428 ± 117 vs. 498 ± 284 copies/µg DNA; p = 0.45) or *P. aeruginosa* (6,152 ± 1,567 vs. 6,540 ± 2,336 copies/µg DNA; p = 0.50). Interestingly, 9 patients with T1D had significantly higher *P. aeruginosa* DNA copy numbers compared with healthy controls (7,762 ± 884 vs. 6,152 ± 1,567 copies/µg DNA; p = 0.035). One patient with T1D had an *S. maltophilia* copy number approximately 2.5-3.0-fold higher than the other subjects. When this individual was excluded from the analysis, a strong correlation between *S. maltophilia* and *P. aeruginosa* copy numbers (r = 0.61; p = 0.003) was observed.

Both *S. maltophilia* (n = 21; r = 0.59; p = 0.005) and *P. aeruginosa* (n = 22; r = 0.55; p = 0.008) flagellin gene copy numbers significantly correlated with age. Age-related correlations were stronger among patients with T1D compared to healthy controls (*S. maltophilia*: n = 11; r = 0.77; p = 0.005 vs. n = 10; r = 0.29; p = 0.43; and *P. aeruginosa*: n = 12; r = 0.79; p = 0.002 vs. n = 10; r = −0.08; p = 0.83).

5.1.3 Analysis of serum antibodies against Pseudomonal bacteria

Serum IgA and IgG antibody levels against *D. acidovorans*, *S. maltophilia*, and *P. aeruginosa* were assessed to quantify systemic exposure to Pseudomonal bacteria.

Within the original study cohort of 14 patients with T1D and 10 controls, we observed no statistically significant differences in serum IgG or IgA levels

between groups. However, there was a trend towards higher IgA concentrations in T1D patients.

Therefore, we assayed serum samples from 200 healthy controls and 200 patients with T1D from the FinnDiane Study. In this larger cohort, T1D patients had significantly higher IgA antibody levels against Pseudomonal bacteria (Figure 7).

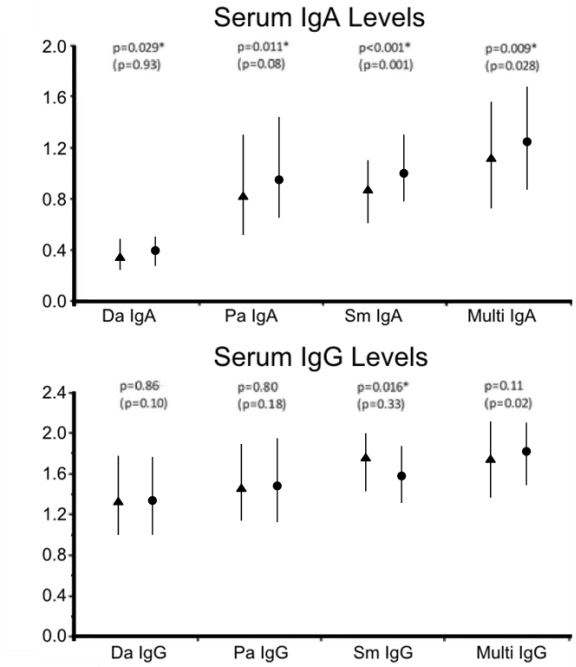

Figure 7 Serum antibody levels against Pseudomonal bacteria. Healthy controls (n = 200) and T1D patients (n = 200) are shown as triangles and circles, respectively. Da, *D. acidovorans*; Pa, *P. aeruginosa*; Sm, *S. maltophilia*; Multi, the pool of Da, Pa and Sm. Data shown are medians with interquartile ranges. A Mann–Whitney U test was implemented to determine significance. p-values within parentheses are adjusted for age, body mass index, and systolic blood pressure. Antibody levels are expressed in arbitrary units.

Tests were adjusted for age, BMI, and systolic blood pressure. After adjustment, group differences in *S. maltophilia* and Pseudomonas-pool IgA/IgG antibody levels remained significant. No significant differences were observed between patients of differing diabetic nephropathy status.

Correlations between CRP and antibody levels were analyzed in a subset of subjects (n=380). Individuals with CRP levels above 10 mg/l were excluded to minimize the possible influence of recent infection. All IgA antibodies correlated positively with serum CRP values (Table 5). In general, correlations between IgA antibodies and CRP were stronger in patients with T1D compared to healthy controls. No significant correlations were observed between serum LPS activity and IgA antibody levels (data not shown). However, a strong correlation was observed between serum LPS and CRP concentrations ($r = 0.221$; $p < 0.001$).

	Serum C-reactive protein			Serum C-reactive protein (Subjects with ≤ 10 mg/l)		
	All	T1D	Control	All	T1D	Control
N	397	199	198	380	193	187
Serum LPS	0.25*	0.22*	0.28*	0.22*	0.20*	0.27*
Anti Da IgA	0.17*	0.21*	0.13	0.15*	0.19*	0.14
Anti Pa IgA	0.15*	0.24*	0.04	0.16*	0.24*	0.08
Anti Sm IgA	0.12*	0.13	0.11	0.11*	0.08	0.14
Pooled IgA	0.15*	0.21*	0.07	0.15*	0.19*	0.1

Table 6 Spearman rank correlation coefficients between serum C-reactive protein, LPS and IgA antibody levels. Da, *D. acidovorans*; Pa, *P. aeruginosa*; Sm, *S. maltophilia*; Multi, the pool of Da, Pa and Sm. * p<0.05

5.2 Study II – High-fat meals induce systemic cytokine release without evidence of endotoxemia-mediated cytokine production from circulating monocytes and myeloid dendritic cells

5.2.1 Analysis of circulating cytokines during fasting and the postprandial period

To better understand postprandial inflammation and its link with circulating LPS, we gave 11 patients with T1D and 11 controls two high-fat meals: breakfast and lunch. From these subjects, we measured circulating cytokines, cytokine production in circulating cells and metabolic parameters such as HDL and triglycerides.

A significant postprandial increase in cytokine concentrations was observed in both T1D patients and healthy controls. In T1D patients, we observed a significant postprandial increase in IL-6, TNF-α, IL-1β, IFN-α, IFN-γ, and MIP-1α levels. Controls showed a significant postprandial increase in IL-6, TNF-α, IL-1β, IFN-α, IL-10, and IL-12 levels. No significant differences were observed between the two groups at either time point. The groups were therefore combined to increase statistical power. After combining, we observed significant postprandial increases in the concentrations of 8 of the 13 innate immunity-derived cytokines: IL-6, TNF-α, IL-1β, IFN-α, IL-10, IFN-γ, IL-12, MIP-1β, and IL-1α (Figure 8, Table 7).

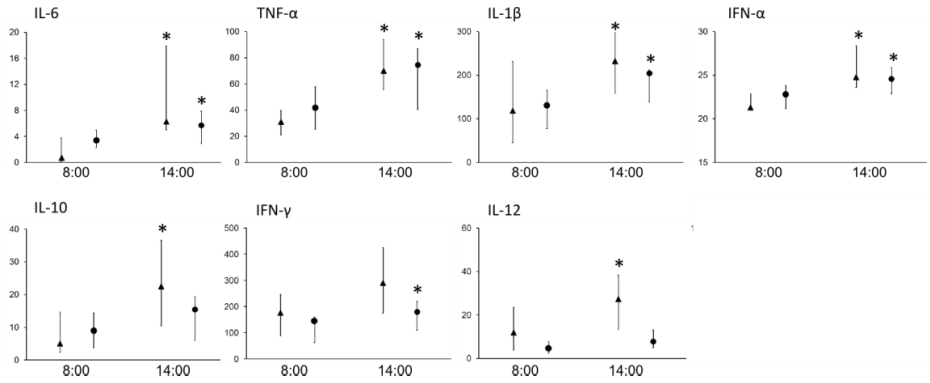

Figure 8 Circulating cytokine concentrations during fasting and the postprandial period. Triangles and circles indicate median values for non-diabetic controls and patients with T1D, respectively. Values represent pg/ml of cytokine. Bars represent the interquartile range. Asterisks indicate a significant difference between 8:00 (fasting) and 14:00 h (postprandial) ($p < 0.05$). All analyses shown differed significantly between 8:00 and 14:00 when the patient and control samples were grouped.

Analyte	T1D 8:00	T1D 14:00	p	C 8:00	C 14:00	p	Grouped P
IL-12p70	4.5	7.8	NS	12	27	0.043	0.016
	(2.4-7.7)	(5-13.2)		(3.9-23)	(13-38)		
LAP	43	46	NS	49	61	NS	NS
	(21-49)	(23-48)		(25-73)	(29-119)		
IFN-γ	145	180	0.047	175	290	0.069	0.007
	(63-161)	(109-220)		(90-245)	(176-426)		
MIP-1β	115	139	0.075	182	203	NS	0.036
	(99-347)	(118-367)		(81-570)	(100-839)		
MCP-1	601	654	NS	565	583	NS	NS
	(537-682)	(573-682)		(528-669)	(532-678)		
IL-10	8.9	15	0.059	5	23	0.038	0.004
	(3.8-14)	(6-19.4)		(2.5-14)	(11-37)		

MIP-1α	728	1318	0.026	2721	2236	NS	NS
	(238-1573)	(399-2922)		(990-5786)	(1067-5269)		
IL-8	54	98	NS	67	62	NS	NS
	(31.3-338)	(45-394)		(14-474)	(28-779)		
ICAM-1	352	324	NS	377	377	NS	NS
	(291-393)	(293-396)		(325-461)	(298-446)		
IL-6	3.4	5.7	0.012	0.7	6.3	0.018	0.001
	(2.3-5)	(2.9-7.9)		(0.2-3.8)	(5.1-18)		
IL-1α	110	146	NS	201	172	NS	NS
	(23-251)	(66-373)		(6-293)	(12-369)		
IFN-α	23	25	0.003	21	25	0.008	0
	(21-24)	(23-26)		(21-23)	(24-28)		
IL-13	142	199	0.063	142	252	0.021	0.003
	(112-189)	(125-225)		(113-182)	(186-380)		
IP-10	56	57	NS	73	92.8	NS	NS
	(46-65)	(52-67)		(55-107)	(65-125)		
IL-4	57	69	NS	55	84	NS	0.039
	(37-69)	(45-73)		(29-89)	(65-109)		
IL-17A	79	90	0.022	119	210	0.036	0.003
	(22-96)	(41-104)		(37-229)	(155-328)		
IL-1β	131	204	0.011	119	232	0.063	0.001
	(78-166)	(141-213)		(46-232)	(160-297)		
TNF-α	42	75	0.017	31	70	0.046	0.002
	(26-58)	(41-87)		(21-40)	(56-94)		
E-Selectin	86	106	NS	159	132	NS	NS
	(59-122)	(64-161)		(76-258)	(69-302)		

Table 7 Medians and interquartile ranges of circulating cytokines. Values represent the median pg/ml of cytokine. Interquartile ranges are in parentheses. p-values represent 8:00 vs. 14:00 within-group p values. Group p values represent 8:00 vs. 14:00 combined group p values.

Interestingly, serum LPS activity levels and circulating cytokine concentrations were not significantly correlated. Postprandial plasma triglycerides showed a significant positive association with circulating IL-12 (r=0.574, p=0.013) and IL-10 (r=0.491, p=0.028) concentrations. Postprandial HDL cholesterol concentrations were inversely associated with circulating IP-10 (r=-0.519, p=0.016) and IL-10 (r=-0.449, p=0.030).

5.2.2 Flow cytometric analysis of IL-6, TNF-α, and IL-1β cytokine production in unstimulated blood monocytes and dendritic cells

To assay inflammation in resting cells during fasting and the postprandial period, we measured the production of three cytokines (IL-6, TNF-α, and IL-1β) in CD14-positive monocytes and CD1c-positive mDCs before and after a series of high-fat meals. These samples, which lacked *in vitro* exogenous LPS stimulation, were designated baseline samples. No significant group differences were observed for fasting baseline monocyte TNF-α, IL-6, and IL-1β production (Figure 9). Diabetic monocytes exhibited lower IL-6 expression compared to healthy controls in postprandial baseline samples (8 vs. 10% expressing, p=0.008). Fasting mDCs from patients with T1D exhibited higher levels of TNF-α (11 vs. 5% expressing, p=0.01) and IL-1β (9 vs. 6% expressing, p=0.02) compared to non-diabetic controls (Figure 8).

5.2.3 Flow cytometric analysis of IL-6, TNF-α, and IL-1β cytokine production in LPS-stimulated blood monocytes and dendritic cells

To assay responsiveness to bacterial endotoxin stimulation during fasting and the postprandial period, we measured the production of three cytokines (IL-6, TNF-α, and IL-1β) in CD14+ monocytes and CD1c+ mDCs after stimulation with 100 ng/ml *E. coli* LPS.

No significant differences were observed between T1D patients and healthy controls for LPS-induced monocyte cytokine production either during fasting

or the postprandial period. During fasting, LPS-stimulated mDCs from patients with T1D exhibited significantly elevated IL-6 (p=0.034) and IL-1β (p=0.040) production compared to healthy controls (Figure 9).

Interestingly, no group differences in cytokine production were observed in postprandial LPS-stimulated mDCs (Figure 9, Table 7). HbA_{1c} negatively correlated with fasting IL-6 and postprandial TNF-α (r=-0.620, p=0.042; and -0.644, p=0.044, respectively) in LPS-stimulated mDCs from T1D patients.

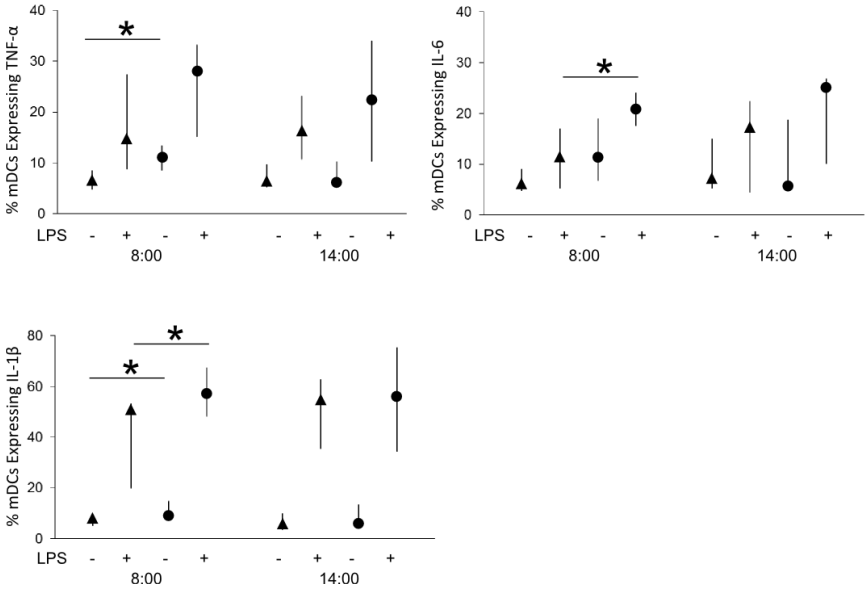

Figure 9 Cytokine secretion during fasting and the postprandial period.

Values represent the median percent of cells expressing the indicated cytokine. Sub indicates the subject type, triangles are controls and circles are T1D patients. Bars represent the interquartile range. * $p<0.05$.

5.3 Study III – Patients with type 1 diabetes exhibit signs of intestinal inflammation

5.3.1 Patient Data

To elucidate intestinal inflammation and IAP, fecal samples were studied to determine the levels of fIAP, calprotectin, immunoglobulins, and SCFAs in 41 non-diabetic controls, 36 patients with type 1 diabetes and normal albumin excretion (AER<20 µg/min or <30 mg/24 h), and 10 patients with type 1 diabetes and macroalbuminuria (AER≥200 µg/min or ≥300 µg/24 h).

The focus of Study III was to investigate intestinal and circulating lipids and inflammation; therefore, participants were analyzed for baseline differences in gender, BMI, lipid profiles, age and diet. No group differences were observed in terms of gender, BMI or circulating lipid profiles. Patients with T1D were slightly older than non-diabetic controls. Patients with macroalbuminuria had higher systolic blood pressure than patients with a normal AER or healthy controls.

No differences were observed between the groups in terms of either pre-study energy intake (control: 2015±517 kcal/24 h; T1D normal AER: 2085±721 kcal/24 h; T1D macroalbuminuria: 1743±640 kcal/24 h) or the percent energy derived from dietary fats (control: 38%; T1D normal AER: 41%; T1D macroalbuminuria: 38%). Energy intake during the 10-h study day was 2600 kcal, of which 50% came from fats.

The primary comparisons investigated in Study III are 1) patients with T1D and normal AER vs non-diabetic controls, and 2) patients with T1D and normal AER vs patients with T1D and macroalbuminuria.

5.3.2 Measurements from fecal samples

Patients with T1D and normal AER had lower levels of fecal intestinal-AP (fIAP) compared with controls (61 [26-221] vs. 131 [73-837] U/l, p=0.01). No correlations between fIAP activity and serum LPS activity, serum lipids, or CRP levels were observed. Patients with T1D and normal AER exhibited a trend toward lower

concentrations of three of the five short-chain fatty acids (SCFAs) studied. Propionate (70 [33-106] vs. 91 [66-116] µmol/g dry weight; p=0.015) and butyrate (39 [23-81] vs. 71 [40-101] µmol/g dry weight, p=0.020) were significantly reduced compared to controls (Figure 10). Moreover, patients with T1D had lower total SCFA levels (414 [234-638] vs. 496 [404-720] mmol/g dry weight, p=0.043), although this was likely driven by propionate, butyrate and valerate. SCFA concentrations were positively associated with carbohydrate intake in normoalbuminuric patients (r=0.483, p=0.003).

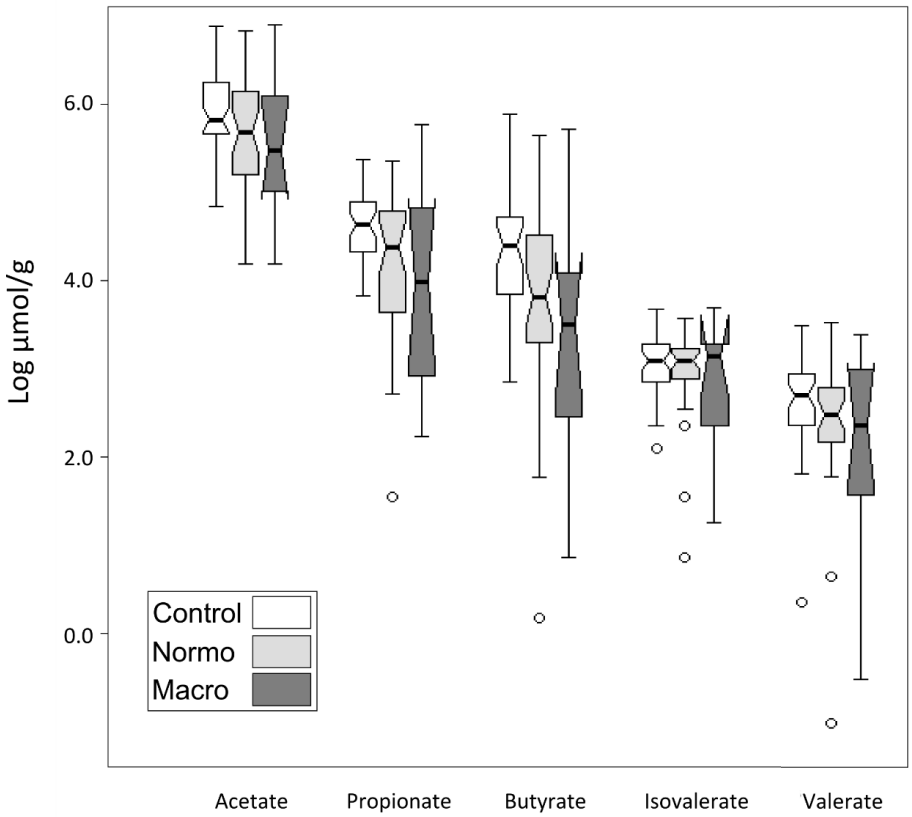

Figure 10 Fecal short-chain fatty acids (SCFA). Total SCFA, propionate, and butyrate levels were lower in patients with T1D and normal AER compared to controls. White boxes: controls; gray boxes: patients with T1D and normal AER; dark gray boxes: patients with T1D and macroalbuminuria. Values are shown as natural logs of the SCFA concentration in µmol per gram. * p<0.05.

Fecal calprotectin is a stable neutrophil-derived biomarker for inflammatory bowel disease (IBD) and has been used in a large number of IBD diagnostic studies[174]. Normoalbuminuric patients have elevated fecal calprotectin levels compared to controls (48 [29-90] vs. 29 [16-59] µg/g, p=0.028) (Figure 11). We designated values greater than 50 µg/g as representing an inflamed gut[174]. Normoalbuminuric patients with T1D were more likely to have gut inflammation than healthy controls (50% vs. 25%, p=0.024). Calprotectin was further elevated in macroalbuminuric patients. Forty percent of macroalbuminuric patients met the clinical criteria for IBD diagnosis: fecal calprotectin >200 µg/g (macroalbuminuric vs normoalbuminuric, 40% vs. 6%, p=0.017).

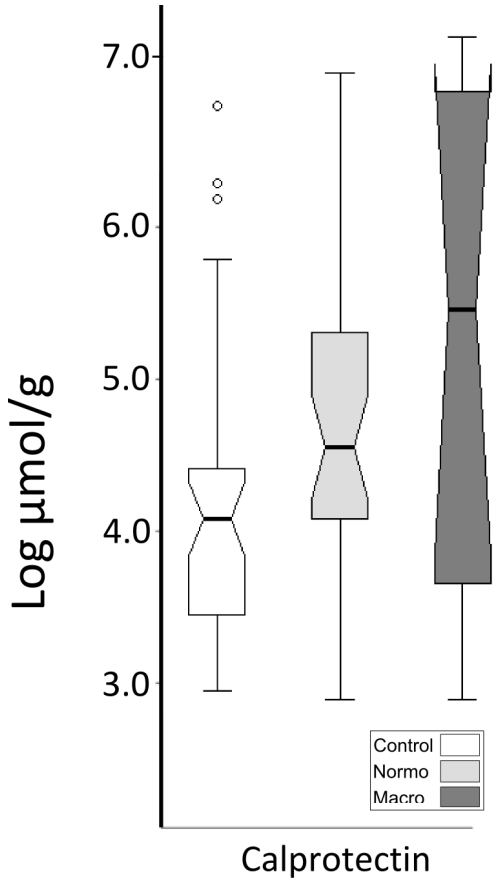

Figure 11 Fecal calprotectin levels. Log-transformed fecal calprotectin levels were higher in patients with T1D and normal AER compared to controls. White boxes: controls; gray boxes patients with T1D and normal AER; dark gray boxes: patients with T1D and macroalbuminuria. Values are shown as natural logs of the SCFA concentration in µmol per gram. * p<0.05.

Fecal immunoglobulins

Indirect ELISA assays were performed to measure total IgA, IgG and IgM levels as well as specific IgA antibodies generated against copper-oxidized LDL or malondialdehyde-(MAA)-modified LDL in fecal extracts. Normoalbuminuric patients demonstrated reduced levels of total IgA and anti-oxLDL compared to controls (total IgA 1.9 [0.7-3.6] vs. 3.4 [1.5-6.9] µg/g wet weight, p=0.015; IgA-MAA 9.2 [3.8-28.8] vs. 24.4 [12.7-57.1] RU, p=0.008; IgA-CuOx 22.1 [12.0-43.4] vs. 56.4 [23.8-112.1] RU, p=0.002). IgG and IgM were similar between both groups. Fecal IAP correlated with total IgA, IgG and IgM levels across groups (correlation determined using all subjects: IgA vs. fIAP r=0.266, p=0.039; IgG vs. fIAP r=0.349, p=0.001; and IgM vs. fIAP r=0.432, p<0.001).

Oral IAP supplementation in mice

C57BL/6 mice were exposed to high-fat diet and various doses (low 1.5-3.0 U ml^{-1}, medium 7.5-15 U ml^{-1}, high 30-60 U ml^{-1}) of calf intestinal phosphatase (cIAP) for 11 weeks, where after caecal and colonic samples were isolated and extracted for subsequent analyses (Figure 12). Compared to the reference group (no_cIAP; n=4), mice receiving the highest oral doses of IAP (high_cIAP; n=8) showed the highest caecal (7.9-fold; 411±106 vs. 3246±694 U/l, p=0.003) and colonic (9.8-fold; 378±234 vs. 3709±1096 U/l, p=0.003) IAP activity.

Oral IAP supplementation increased the caecal (9.3-fold; 4.8±2.2 vs. 44.5±15.0 µg/ml, p=0.003) and the colonic (3.1-fold; 5.9±2.6 vs. 18.5±9.7 µg/ml, p=0.014) content of total IgA, whereas total IgG and total IgM levels were unaffected. IAP activity levels correlated with the caecal (r=0.82, p<0.001) and the colonic (r=0.67, p<0.001) content of total IgA. However, IAP activities showed no correlations with total IgG or total IgM concentrations.

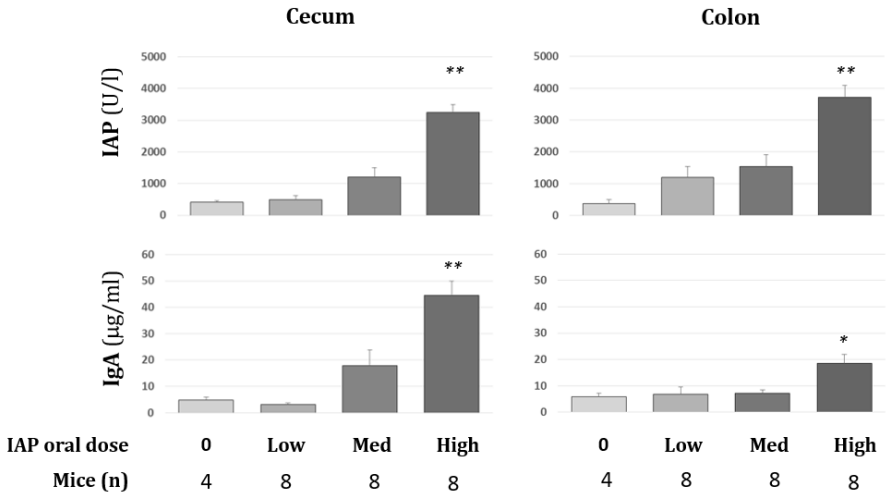

Figure 12 Intestinal IAP activity and total IgA levels in mice after oral IAP supplementation. C57BL/6 (n=28) mice were exposed to high-fat diet and various doses of cIAP: no cIAP (n=4), low cIAP dose 1.5-3.0 U/ml (n=8), medium cIAP dose 7.5-15 U/ml (n=8), high cIAP dose 30-60 U/ml (n=8) for 11 weeks. IAP activity and total IgA levels are shown for caecal and colonic samples. Histogram data is presented as mean ± standard error of mean. Shown p-values are obtained from the Jonckheere-Terpstra trend test. * p<0.01, ** p<0.001.

5.3.3 Serum sample measurements

Serum Alkaline Phosphatases

Alkaline phosphatase was quantified every 2 h during the investigation day. No group differences were observed for total alkaline phosphatase activity. Total alkaline phosphatase (total-AP) positively correlated with CRP in controls (r=0.455, p=0.006) and normoalbuminuric patients (r=0.366, p=0.036). Total-AP and triglycerides also correlated in normoalbuminuric patients (r=0.399; p= 0.021). These associations were driven by the liver isoform, which accounted for nearly 50% of all alkaline phosphatase in circulation. Liver alkaline phosphatase correlated with CRP (control r=0.460, p=0.003; normoalbuminuric T1D r=0.364, 0.037) and triglycerides (normoalbuminuric T1D r=0.472, p=0.006). To assess the daily accumulation of serum factors, the area under the curve (AUC) was calculated for each patient and for each analyte based on measurements from the six samples taken during the day.

An analysis of alkaline phosphatase isoforms found no changes in bone, liver, or macro isoforms over the course of the investigation day. Interestingly, intestinal isoform

(sIAP) levels showed a significant postprandial increase (Figure 13). At all time points and over the course of the day (AUC), normoalbuminuric patients exhibited elevated levels of sIAP compared to controls (Figure 13, AUC 87.7 [17.9-202.2] vs. 21.6 [8.1-85.2]; p=0.007). ABO blood group and FUT2 secretory phenotype frequencies were similar between groups, and the difference in sIAP remained significant after controlling for these factors. These frequencies were tested due to the role of blood groups and the FUT2 genotype in IAP production and secretion. In controls, sIAP-AUC was negatively associated with postprandial endotoxemia (LPS-AUC r=-0.454, p=0.005) and positively associated with HDL-lipoprotein metabolites (HDL-cholesterol-AUC r=0.404, p=0.011; ApoA1-AUC r=0.422, p=0.007; CETP-AUC r=0.464, p=0.003). Fecal IAP showed no association with sIAP at any time point or with sIAP-AUC.

Macroalbuminuric patients demonstrated elevated levels of the liver isoform during fasting compared to normoalbuminuric patients (36 [31-46] vs. 27 [16-35], p=0.041), and this elevation continued throughout the day (AUC 330 [273-408] vs. 214 [150-301], p=0.013). In normoalbuminuric patients, fasting liver-AP activity was strongly associated with obesity (BMI r=0.541, p=0.001), triglycerides (r=0.472, p=0.006) and percent body fat (r=0.449, p=0.009). No group differences were observed for total-AP, likely driven by the lack of variance in the bone isoform, which represents nearly 50% of total-AP and was comparable between groups.

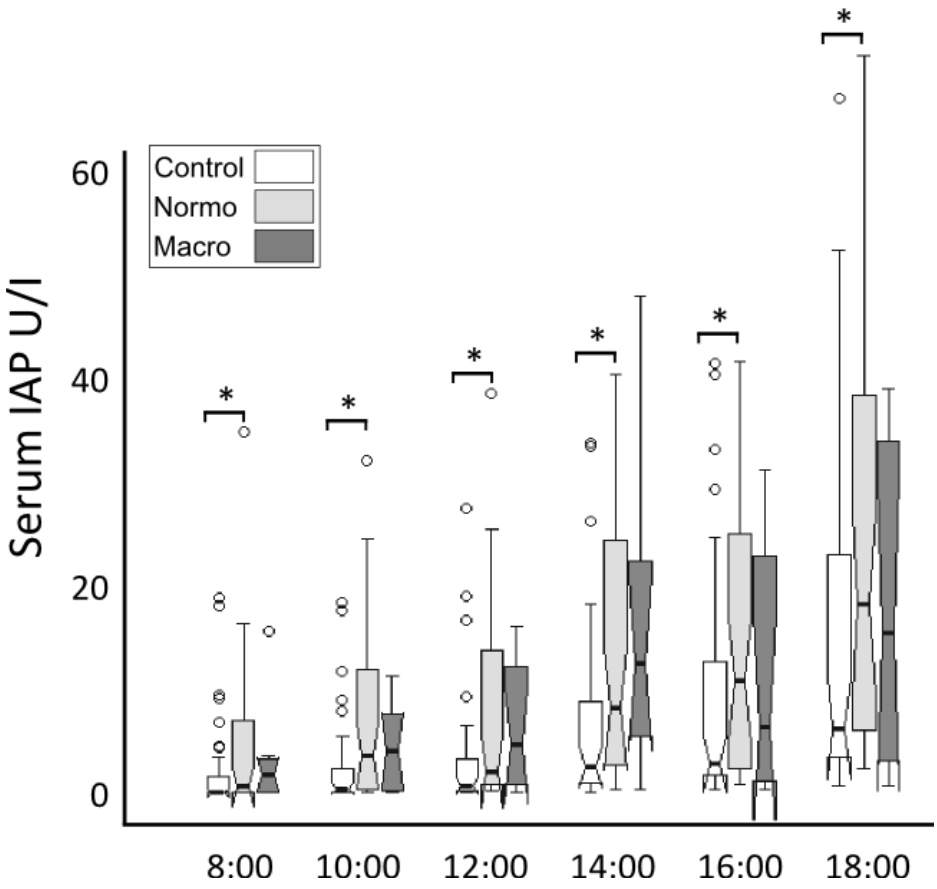

Figure 13 The effects of three high-fat meals on serum intestinal alkaline phosphatase activity (sIAP). Patients with T1D and normal AER had elevated serum IAP levels compared to controls. Meals were given at 8:00, 12:00, and 16:00. White boxes: controls; gray boxes: patients with T1D and normal AER; dark gray boxes: patients with T1D and macroalbuminuria. * $p<0.05$.

6 Discussion

LPS and methylated CpG DNA are two bacterial remnants that are recognized by the human innate immune system. Upon ligation of these bacterial remnants, human cells release inflammatory mediators, which in turn contribute to the development of many diseases, such as atherosclerosis, as well as diabetic complications.

The presence of elevated levels of bacterial remnants such as LPS in patients with T1D has become evident, but the source and consequences have not been well characterized[7]. Determining the bacterial species and strains that give rise to the LPS that is detected by the LAL assay would facilitate a better understanding of the LPS source and the development of therapeutics or lifestyle interventions to decrease the amount of active LPS in circulation. The most likely sources for the LPS that is commonly observed in circulation include the oral cavity and gut.

Studies I, II and III were performed to better understand the role of circulating bacterial components and their potential associations with inflammatory responses in type 1 diabetes.

6.1 Identification of bacterial DNA – clues regarding the source of bacterial remnants

Data from both serum-derived bacterial DNA and serum antibodies has demonstrated patients with T1D experience increased exposure to or infections with Pseudomonal bacteria compared with the general population (Study I).

The driving force behind the identification of bacterial DNA in circulation is the desire to elucidate the possible routes of entry for circulating LPS, based on the hypothesis that isolated DNA enters the bloodstream together with LPS.

One potential route of entry for circulating bacterial remnants is the intestinal tract. Patients with T1D have altered intestinal barrier function, which may lead to increased gut permeability[175]. As shown in Study I, a significant proportion of the identified DNA was actually derived from environmental bacteria and pathogens. Therefore, it is highly unlikely that circulating bacterial DNA is derived from the intestine given that less than 2% of all identified bacterial species are gastrointestinal tract-specific. These

data align with another study in which healthy subjects were found to have only trace amounts of bacterial DNA derived from gut bacteria[176].

The second potential route of entry for bacterial remnants into circulation is the oral cavity. Indeed, patients with T1D suffer from an increased rate of periodontitis[107]. Moreover, in the general population, chewing, tooth brushing and other dental procedures cause bacteremia and endotoxemia[108,177,178], suggesting that the bacterial DNA found in circulation does not come from the gastrointestinal tract but rather an alternative route of entry.

The most prevalent bacteria were of Pseudomonal origin, which belongs to the phylum proteobacteria. Notably, patients with T1D had significantly higher frequencies of *S. maltophilia* (a Pseudomonal bacteria) clones in their serum compared to healthy controls. The overrepresentation of proteobacteria may be attributable to the overrepresentation of proteobacterial colonization or infections. Interestingly, some of the identified bacteria are involved in oral infections, which cause bleeding gums and thereby may increase the amount of bacterial remnants in circulation[179,180].

However, differences in prevalence may also be related to differences in host clearance mechanisms or increased resistance to proteolytic degradation based on bacterial or DNA structure within that phylum. Interestingly, immune deficiencies leading to an impaired response to proteobacteria have previously been shown to cause intestinal inflammation in animal models [181], a phenomenon that is more common in patients with T1D according to Study III.

However, there was no correlation between LPS activity and either bacterial DNA composition or antibodies against identified bacterial species, casting doubt on the hypothesis of shared entry; however, this phenomenon may also be attributable to differences in clearance mechanisms and half-lives. Indeed, it is entirely plausible that bacterial LPS enters circulation through mechanisms unrelated to the origin of circulating bacterial DNA.

6.2 High-fat diets: a framework for investigating the origins and consequences of acute postprandial endotoxemia and inflammation

Multiple high-fat meals were given to 52 patients with T1D and 41 healthy controls to investigate the effects of dietary fat on intestinal inflammation, postprandial endotoxemia and its subsequent systemic inflammation in the context of T1D. Fecal samples were collected before and after the exam, and blood samples were taken every 2 h from 8:00 until 18:00. A number of studies were then conducted on the whole cohort and cohort subgroupings. Studies II and III in this thesis are among the studies conducted on the high-fat diet cohort.

According to earlier studies, fatty diets trigger metabolic endotoxemia and systemic inflammation[11,138]. Although the precise mechanisms of this postprandial endotoxemia and inflammation are not clear, it has been suggested that increased chylomicron production triggered in response to a high-fat diet increases LPS transport from the gut into circulation[10,139,140]. Given the established link between the progression of diabetic kidney disease and elevated levels of endotoxins and inflammatory markers, the pro-inflammatory response to dietary fat may play a role in the pathogenesis and progression of diabetic complications[3-5,7,182]. Moreover, these factors likely become pathogenic if anti-inflammatory counter-regulatory mechanisms fail. Indeed, *in vitro* experiments in isolated cells have found increased TLR4 expression and responsiveness in cells from patients with T1D[61,78,183].

In Study II, we sought to investigate cellular cytokine secretion in postprandial immune cells to elucidate the cellular origins of postprandial inflammation and confirm the dysregulation of LPS reactivity of diabetic immune cells in an *ex vivo* setting.

Our *ex vivo* stimulation tests in whole blood were unable to confirm reports of increased monocyte cytokine secretion in response to LPS. One potential reason for this is the presence of different amounts of autologous serum factors such as LPS-binding proteins or serum lipids in the peripheral blood of patients with T1D. Indeed, patients with T1D had elevated fasting serum HDL cholesterol levels, which are known to buffer LPS-induced inflammation[184]. However, our data did not suggest any effects of serum HDL cholesterol on cytokine production in circulating innate immune cells.

The experimental design employed in the fat tolerance study facilitated the investigation of systemic and intracellular inflammation to elucidate the mechanisms of postprandial endotoxemia and inflammation. Study II found that acute, high-fat meals increase circulating cytokines but have no effect on serum LPS activity levels or cytokine production in circulating immune cells. Thus, high-fat meals increase circulating cytokines and chemokines independent of postprandial LPS and circulating innate immune cells. Therefore, postprandial inflammation is likely regulated at the tissue level in response to fats or locally accumulated endotoxins. Indeed, evidence from murine models has suggested that postprandial inflammation may be derived from the intestine, liver or adipose tissue [185,186].

Interestingly, unlike previous studies using isolated cells, study II found no difference in the LPS responsiveness of circulating cells in whole blood from patients with T1D compared to controls. This discrepancy with previous reports may be attributable to endogenous factors within the blood that buffer the hyperresponsiveness observed in cells isolated from patients with T1D.

6.3 High-fat diets: a framework for investigating intestinal homeostasis and metabolic disturbances in type 1 diabetes

The high-fat diet trial resulted in a study that was used for several projects. The first study, utilizing most of the participants, found that controls showed only a modest increase in postprandial LPS activity. However, patients with T1D experienced only a modest decrease in LPS activity in response to multiple fatty meals[14]. This is in contrast with earlier, smaller studies in humans reporting increased postprandial LPS activity[11,138]. The discrepancy with previous studies may be attributable to the effects of postprandial lipemia in the LAL assay. In our studies, we corrected for the distorting effects of serum triglycerides by subtracting the sample background from the absorption. The uncorrected lipemic solutions yielded higher perceived activity levels than non-lipemic solutions. Therefore, it is possible that earlier reports on postprandial endotoxemia were artefactual, primarily driven by lipid-induced nonspecific signals.

Lassenius[187] (2012) reported increases in postprandial inflammation despite the lack of significant postprandial endotoxemia. Moreover, there was no correlation between

serum LPS activity levels during the day and the inflammatory markers IL-6, SAA, CRP or sCD14. This agrees with Study II and further underscores the decoupled nature of postprandial LPS and concomitant inflammation. Therefore, it is likely that postprandial LPS exhibits attenuated toxicity. This could be the result of LPS-detoxifying factors such as alkaline phosphatases or the lipolysis-induced postprandial generation of HDL. Study III, therefore, investigated intestinal inflammation and alkaline phosphatase, which are factors that might contribute to systemic inflammation, LPS internalization, and LPS toxicity.

Study III reported inflammation in the intestine in patients with both uncomplicated and complicated T1D. All patients with T1D showed significantly elevated calprotectin levels, and 40% of the patients with diabetic nephropathy had levels above the diagnostic threshold for inflammatory bowel disease.

Calprotectin is a neutrophil-derived factor that has been used both for the diagnosis of inflammatory bowel disease and to distinguish between inflammatory and functional bowel disorders[188,189]. It has previously been shown that elevated levels of fecal calprotectin reflect neutrophil migration to the intestinal lumen[190]. In a separate study using the same participants, patients with T1D exhibited a blunted increase in circulating neutrophils in response to high-fat meals[14]. Taken together, the elevated calprotectin levels and blunted neutrophil response suggest that patients with diabetes have an inflamed intestine with neutrophil infiltration. However, further studies are needed to clarify the role of neutrophils in intestinal inflammation in T1D.

Intestinal inflammation was further evidenced by diminished levels of fecal IAP in stool samples from patients with T1D. This also agrees with previous reports demonstrating decreased fecal IAP in the stool samples of patients with inflammatory bowel disease and celiac disease[161,191]. Given the role that IAP plays in the detoxification of LPS, it is possible that gut-derived bacterial LPS from patients with T1D is more toxic. The LPS-detoxifying role of IAP and other alkaline phosphatases may explain the seemingly reduced inflammatory capacity of LPS observed in Study II.

The prevalence of inflammatory bowel disease in patients with T1D is a growing area of interest, and one recent study found the prevalence of inflammatory bowel disease to be 6-fold higher in patients with T1D compared to the general population[152]. Notably, our data suggest that the similarities with clinical inflammatory bowel

disease extend well beyond fecal calprotectin. Indeed, our data show lower fecal IAP activity, lower levels of butyrate and reduced overall concentrations of short-chain fatty acids. These results align with what has been observed in inflammatory bowel disease and further suggest that the intestine plays an integral role in inflammation in the context of T1D[160,192,193]. Furthermore, the heightened inflammatory state coupled with a more permeable gut might contribute to increased translocation of inflammatory mediators from the gut into circulation.

In contrast to what was observed in fecal samples, patients with T1D were found to have aberrantly high levels of circulating IAP. While IAP is released directly into circulation by the enterocytes, it also enter circulation via increased gut permeability caused by intestinal inflammation[153,155,194]. Alternatively, the release of IAP into circulation may be part of a compensatory mechanism intended to resolve ongoing endotoxin-mediated inflammation. Indeed, serum IAP activity levels were associated with decreased LPS-AUC in healthy controls, supporting its role in LPS detoxification[155].

7 Summary and Conclusions

The present studies concentrated on endogenous and exogenous factors that influence inflammation, which represents an important step in the development of diabetic kidney disease. The investigated factors were bacterial LPS and DNA, the postprandial milieu, and markers of intestinal inflammation and homeostasis.

7.1 Study I

In Study I, the proportion of isolated DNA in T1D patients significantly differed from that of healthy controls, and this was reflected in a multistereotype antibody analysis. Moreover, we reported an overrepresentation of pathogenic and environmental flora with a near absence of normal intestinal flora. Further investigation into the most prevalent flora revealed them to be involved in oral infections, raising the possibility that bacterial DNA enters into circulation through the oral cavity. Specifically, oral infections may cause bleeding gums and thereby allow more bacterial components to enter into circulation. Notably, no correlation was observed between LPS activity and either bacterial DNA composition or antibodies against identified bacterial species. It is therefore plausible that the entry mechanisms and points of entry of LPS and bacterial DNA are different.

These observations build on the body of knowledge regarding immunological dysregulation and differential infection rates and outcomes in patients with T1D.

7.2 Study II

Study II was the first study to focus on the effects of multiple physiological high-fat meals on cytokine production in circulating innate immune cells. Furthermore, it was the first to investigate postprandial inflammation and cellular activation in the context of T1D. Multiple high-fat meals increased circulating cytokines and chemokines independent of postprandial LPS. Moreover, this increase was not mediated by circulating innate immune cells. Therefore, postprandial inflammation is not induced by increased amounts of systemic LPS and is likely indicative of diet-induced, tissue-specific inflammation. Based on these results, postprandial inflammation may derive

from intestine, liver or adipose tissue inflammation. Study II represents a paradigm shift in our understanding of early postprandial inflammation and has sparked further studies into the source and mechanisms of postprandial inflammation.

7.3 Study III

In Study III, patients with T1D demonstrated significantly elevated levels of calprotectin coupled with aberrantly low levels of fecal IAP activity as well as butyrate and overall short-chain fatty acid concentrations. Taken together, these data point to a general state of intestinal inflammation in patients with T1D. Gut inflammation explains the commonly observed postprandial systemic inflammation through the release of inflammatory mediators into circulation. Study III also highlighted the overlap of T1D with inflammatory bowel disease, with even higher intestinal inflammation found in macroalbuminuric patients with T1D. Importantly, the results in Study III suggest that oral IAP or butyrate supplementation may suppress low-grade intestinal inflammation in patients with diabetes. Further studies are needed to better understand the role of intestinal inflammation in the progression of diabetic kidney disease.

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